BIOTRANSFORMATION OF SELENIUM AND ARSENIC IN INSECTS: ENVIRONMENTAL IMPLICATIONS

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By

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Keywords: insects, selenium, arsenic, biotransformation, chemical form, X-ray absorption spectroscopy

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ABSTRACT

Living organisms constantly respond to changing environmental conditions, and some changes can be far from optimal for many organisms. Insects represent the majority of species in many ecosystems and play an important role in bioaccumulation and biotransformation of environmental contaminants such as selenium and arsenic. Some insectivorous predators feeding on these insects are highly sensitive to such elements resulting in reduced growth, reproductive failures and low population numbers. The mechanisms of selenium and arsenic uptake through the food chain are poorly understood. The determination of chemical speciation is a prerequisite for a mechanistic understanding of a contaminant's bioavailability and toxicity to an organism. Synchrotron-based X-ray absorption spectroscopy was used to identify the chemical form of selenium and arsenic in insects in both the field and laboratory conditions. Insects living in streams near Hinton, Alberta affected by coal mine activities were examined for selenium speciation. Results showed higher percentages of inorganic selenium in primary consumers, detritivores and filter feeders than in predatory insects. Selenides and diselenides constitute a major fraction of selenium in these insects. In another field setting, speciation of selenium was studied in insects attacking selenium hyperaccumulating plant *Astragalus bisulcatus*. The effect of selenate and arsenate alone and the combined effects of selenate and arsenate on insects and parasitoids were monitored using a laboratory-reared moth (*Mamestra configurata*). Hosts receiving selenium biotransformed selenate to organic selenides and diselenides, which were transferred to the parasitoids in the third trophic level. Arsenic fed larvae biotransformed dietary arsenate to yield predominantly trivalent arsenic coordinated with three aliphatic sulfurs. Larvae receiving arsenate used a novel six-coordinated arsenic form as an excretory molecule in fecal matter and cast skin. X-ray absorption spectroscopy imaging with micro X-ray fluorescence imaging on selenate and arsenate fed larvae revealed highly localized selenium and arsenic

species, zinc and copper within the gut. The results provide insights into how the insects cope with their toxic cargo, including how selenium and arsenic are biotransformed into other chemical forms and how they can be eliminated from the insects. The implication of selenium and arsenic species in the diet of predators and detritivores is discussed.

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1. INTRODUCTION

1.1 Insects and Their Ways

Insects are enormously successful in terms of species richness and abundance. The number of known species of insects is approximately one million, well above the number of all other known species of animals (1). After 350 million years of constantly adapting to new and changing environmental conditions (1), insects have established themselves in about every terrestrial, freshwater and near shore marine habitat. Some species are found deep underground and some several hundreds of meters in the atmosphere. Some species can survive several months exposed to below-freezing temperatures, whereas other species develop in fairly hot habitats (1, 2). This extreme diversity in habitats is reflected by a similar diversity in the shapes, sizes, colors, behaviors and food preferences of insects. They can vary in size from 0.2 mm to 300 mm long (1), and in shape from resembling a thorn to mimicking a leaf, twig or another insect.

Many insects are extremely valuable to humans. By their pollinating activities, they aid in fruit and seed production in agricultural crops. They are producers of honey, beeswax, silk and other products of commercial value (2). Insects serve as food for many fish, amphibians, birds and other organisms including humans and also perform valuable services as scavengers (1). They aid in control of noxious weeds and harmful animals. Insects have been useful in medical treatments and in scientific research to solve problems in heredity, evolution, physiology, ecology and environmental quality. A few insects are harmful and damage cultivated crops, stored food, clothing and structures, and some insects transmit diseases that seriously affect the health of humans, other animals and plants (2). Interactions of insects with humans and other organisms are important in

functioning of ecosystems and maintaining ecological balance. Disruption of any of these activities can have significant effects on the environment (3).

1.2 Risks of Metals and Metalloids

In general, most elements are metals. They are typically shiny, good conductors of heat and electricity, have a high density, and melt at high temperature. Nonmetals are different from metals in that their surface is dull and they are poor conductors of heat and electricity. As compared to metals, they have low density and low melting points. Although there is no rigorous definition for the term metalloid, in general, metalloids have properties of both metals and nonmetals. Metalloids form amphoteric oxides, and behave as semiconductors.

Contamination occurs when undesirable forms of elements occur in sufficient quantities to harm the environment. Although natural geological deposits containing metals and metalloids occur around the world and rock weathering and volcanic activities may release some metals and metalloids into the environment (4, 5), contamination can also be associated with anthropogenic sources. Human activities have greatly increased the fluxes of many potentially toxic metals and metalloids into aquatic and terrestrial ecosystems affecting insects and other wildlife (6, 7).

Trace concentrations of some metals and metalloids are required for normal functioning and development of animals; moderate concentrations can be stored in the body and homeostatic functions are maintained; high levels can result in toxic effects (4). The concentrations required for these effects may depend on the element and its chemical form. The ability of an animal to regulate these elements may have profound effects on the ecosystems. An organism exposed to toxic metals or metalloids will encounter different levels of responses. At sub individual levels, molecular, cellular and physiological functions may be affected. These responses are integrated at the individual level and are commonly expressed as effects on

survival, growth and reproduction, which together determine the effects at the population and ecosystem levels (6, 7).

Organisms subjected to metal pollution usually have fewer species, lower diversity and lower biomass than organisms in pristine environments (8-10). There is also a shift in species composition from sensitive to tolerant species (10). The sensitivity of insects to metals and metalloids has been widely studied (10-12). Highly sensitive species may face low population numbers or local extinction. Insects sensitive to metals and metalloids, such as mayflies, were the first to disappear and the last to reappear in a copper contaminated site even when the contamination levels were low (13). However, many insects are capable of tolerating elevated levels of metals and metalloid contaminants from the environment (7, 13). The consequences of such tolerance may be the presence of elevated levels of potentially toxic metals or metalloids in insects affecting other sensitive animals that depend on these insects as major sources of food.

Due to their abundance and complex lifecycles, insects may play an important part in geographically distributing potentially toxic heavy elements. When life stages of insects occur in different habitats, movement of metals or metalloids to a new habitat may occur. Flying insectivores such as swifts, swallows, and bats, some of which are threatened and endangered species, forage over water and consume emerging adult insects, which have aquatic larval and nymphal stages (6, 12). Hence, immature insects living in water can be important carriers of metals and metalloids from sediments into the water column and to the terrestrial food chain. Similarly, immature stages of terrestrial insects living on plants or soil containing elevated concentrations of metals and metalloids may carry these elements long distances when they emerge as flying adults. Also, metals and metalloids can cross-geographical boundaries by insect migration.

The qualitative and quantitative characterizations of toxic effects are essential for an evaluation of potential hazards posed by a particular element (14).

It is also important to understand the mechanisms responsible for the manifestation of toxicity such as how the element enters an organism, how it interacts with the body, and how the organism deals with the element.

1.3 Acquisition of Metals and Metalloids by Insects

1.3.1 Mode of Entry

Routes of entry into an organism as well as duration and frequency of exposure are important determinants of toxicity of an element. Possible routes of uptake of metals and metalloids into animals include ingestion, inhalation, and surface absorption (10). Therefore, the elements may cross the integument, respiratory organs and alimentary canal of the insect to exert their deleterious effects. An element may pass through a number of cells, such as epithelium of the skin, the thin cell layers of the respiratory organs or the gastrointestinal tract. Other barriers include membranes of single cells, mitochondria and cell nuclei. Possible mechanisms of uptake include adsorption, passive diffusion, active transport and facilitated diffusion (15) Metal dissolved in water passes into the integument of an insect through adsorption. Depending on the solubility of the element, it may be transported passively involving simple diffusion or by special transport mechanisms. Small water-soluble molecules diffuse across membranes through aqueous pores (4), whereas lipid soluble molecules diffuse across the lipid areas of the membrane. Some compounds are too large to pass through aqueous pores or too insoluble in lipids to diffuse across the lipid domains of membranes. They are often transported by special transport mechanisms such as active transport against the concentration or electrochemical gradients (4).

1.3.2 Distribution of Elements into Target Sites

An element absorbed into the body is distributed to different areas including one or more sites where it causes damage. This site can be a target organ, target tissue or target molecule. These targets may be macromolecules such as nucleic acids (especially DNA) and proteins or small molecules such as membrane lipids, enzymes and cofactors (4). Metallothionines are a class of relatively small proteins, which contain sulfur-rich cysteine amino acids. These metallothionines have high-affinity to bind metals and metalloids (4). Some elements accumulate in adipose tissues and exoskeleton (15, 16) in insects where they do not exert significant toxic effects. Such storage mechanisms may reduce the availability of the elements to the target site at least temporarily. Therefore, identification and characterization of the target site in a particular organism are important in understanding the impact of toxic elements.

Toxic effects are not produced by metals and metalloids unless they reach the target site at a concentration and for a length of time sufficient to produce a toxic manifestation. The measurement of the level of toxicant within the target organ or tissue may reflect the recent exposure or long-term past exposure, depending on the retention time of the element in that organ or tissue and the speciation of the element at the target site. A critical determinant of retention of a metal or metalloid is its biological half-life, that is, the time it takes for the body or organ to excrete half of the accumulated amount. The biological half-life varies according to the element, its chemical form and organ or tissue concerned (4).

1.3.3 Absorption

The gastrointestinal tract is one of the most important sites for absorption of toxicants (16, 17). The gastrointestinal tract of an insect is a tube, usually somewhat coiled, with three main regions: the foregut or stomodaeum; the midgut or

mesenteron; and the hindgut, or proctodaeum (Figure 1.1). Both foregut and hindgut are derived from ectodermal tissue and are lined internally by a thin layer of cuticle. This cuticle is shed at each molt along with the outer exoskeleton (17). Many insects have mouthparts, which consist of chewing mandibles that cut, crush or macerate food material and force them into the mouth (17). In sucking insects, liquid food is taken through the beak (17).

The midgut is the primary site of digestion and absorption in the alimentary canal (18). The midgut epithelium of some arthropods is increasingly recognized as an important toxicologically relevant organ system. In many species, the midgut epithelium and the food are separated by peritrophic matrix (Figure 1.1), which is a non-living, permeable network of chitin and protein secreted by the epithelium (19). The majority of the functions of the peritrophic membrane depend on its permeability, which includes prevention of nonspecific binding of material into midgut membrane (midgut). The peritrophic matrix is critical to protect the arthropod midgut from toxins, microbial infections, digestive enzymes, and physical trauma (19-21). Exposure to metals compromised the integrity of the peritrophic matrix of mosquito larvae (22). In this study, the effect on the peritrophic matrix was metal and dose dependent, and the concentrations of metals used were well within those found in contaminated aquatic ecosystems and aquifers.

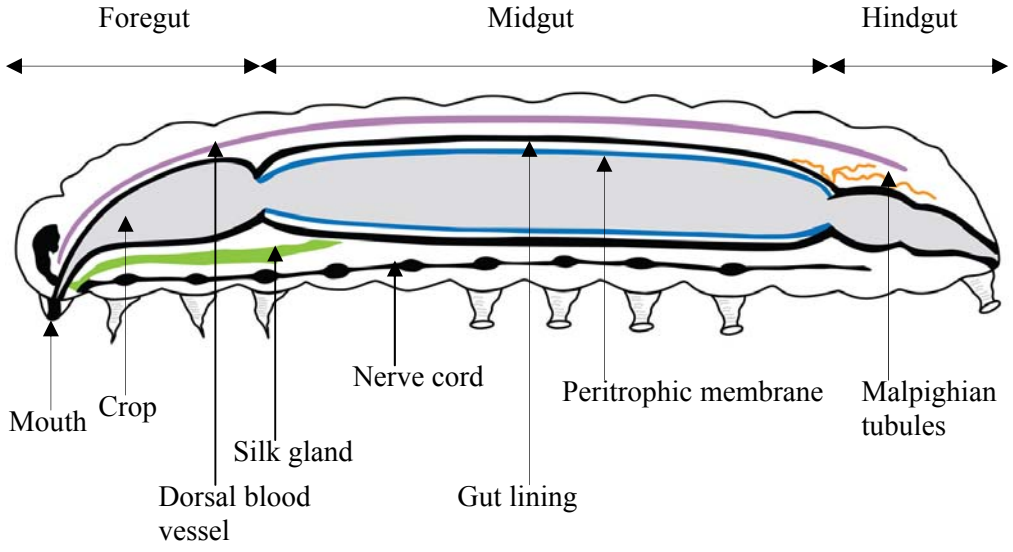

Figure 1.1 Internal anatomy of larval Lepidoptera

1.3.4 Storage and Excretion

Once inside the insect, a metal can be either retained inside the insect or excreted. Insect may have specific storage sites, which minimize its impact on the organism. However, it could also be retained in a non-specific way which, depending on the nature of the chemical form, may or may not cause toxic effects. Insects are able to store metals in the exoskeleton (13). Metals have been found in the malpighian epithelia of housefly [*Musca*] (23), the midgut epithelia of fruit fly [*Drosophila*] (24) and digestive epithelium and fat body of midges [*Chironomus*] (25).

These storage mechanisms minimize the bioavailability of toxic elements making them less toxic to the insect. Retention combined with a chronic exposure

may lead to bioaccumulation in which the insect contains a higher concentration of the toxicant than what it feeds on. Some insects are able to bioaccumulate relatively large amounts of metals and metalloids (25-28) and become significant sources of dietary metals and metalloids to their predators.

Insects are able to remove toxic elements from the body and return them to the environment by excretion. Various organs participate in the excretory process. Insect exoskeleton is associated with binding of metals and metalloids. Elements bound to exoskeleton may be lost with cast skin (exuvia) upon each molting (27, 29, 30).

Malpighian tubules in insects that arise at the anterior end of the hindgut have excretory functions analogous to the mammalian kidney. Metals can be extracted by malpighian tubules (17) and emptied into the gut and then removed in fecal matter (frass). Unabsorbed metals and metalloids passing through the gut may also be excreted in frass. Volatilization of elements from the body (27, 31) and secretions in honeydew and wax (32) and presumably silk and spit could also play a role in excretory process.

1.3.5 Biotransformation

Once taken up, biotransformation of the element may take place, in which the chemical form of the element is changed. Biotransformation of the element may alter its biological effects, resulting in the formation of less toxic or more toxic forms. Many organisms are capable of transforming metals and metalloids in the abiotic environment into different chemical species (chemical forms). Knowledge of the chemical speciation of contaminants is essential for predicting their properties such as stability, mobility, toxicity, and potential bioavailability to organisms (14). There are different detoxification pathways depending on the chemical nature of the toxic substance.

1.4 Selenium and Arsenic in the Environment

Selenium and arsenic are classified as metalloids that exhibit properties of both metals and non-metals (14). Selenium and arsenic are distributed naturally in the environment by geological weathering, volcanic activities and by accumulation in biological material (14). However, human industrial activities may greatly influence the worldwide distribution of these elements. As a result, there is a growing interest in the environmental transport and toxicity of elevated levels of selenium and arsenic.

1.4.1 Essentiality, Toxicity and Chemical Forms of Selenium in Organisms

Elemental selenium occurs in various forms of allotropes ranging from a grey metallic to a reddish glassy appearance (14). Elemental selenium is characterized by atomic number of 34 and atomic weight of 78.96 and some chemical properties of selenium are similar to those of sulfur (14). Selenium is an essential micronutrient in animals, but can become toxic at slightly higher levels (4, 28). There is a small margin of safety between nutritional level and toxic level of selenium (33). Toxic effects have been described in animals living in various seleniferous areas (28, 33).

Some plant species are able to accumulate selenium to levels more than 1000 $mg.kg^{-1}$ dry weight (34, 35). When consumed by livestock, these plants may cause a disease described as blind staggers. Symptoms include impaired vision, depressed appetite, and a tendency to wander in circles which may progress to various degrees of paralysis and death from respiratory failure (4). A more chronic syndrome described in livestock and horses is alkali disease, which is characterized by deformities, shedding of hoofs, loss of long hair and erosion of joints of long bones (4). In birds, selenium can cause fewer eggs to hatch, a higher death rate of chicks, abnormal developments, reproductive failures and suppression of the

immune system (33). Selenium has produced loss of fertility and congenital defects and is considered embryotoxic and teratogenic on the basis of animal experiments (36).

Deficiency of selenium in lambs and calves produces "white muscle disease", a form of muscular dystrophy. The most extensively documented deficiency of selenium in humans is Keshan disease. This is a cardiomyopathy first discovered in Keshan county in the People's Republic of China in 1935 (37). The disease is associated with degeneration and necrosis of myocardial fibers and their replacement by fibrous scars. (37). Deficiency of selenium produces liver necrosis in rats, bleeding disorder in poultry, and cellular necrosis in liver, kidney, skeletal and heart muscle in mice. In each of these symptoms health was improved by adding selenium to the diet (37).

Selenium plays an important role in the physiology of insects (38, 39). Selenium deficiency has been shown to reduce survival and fertility of the fruit fly, *Drosophila* sp. (38). In that study, selenium levels in the diet up to 10 $nmol.kg^{-1}$ were sufficient to maintain a normal life while levels above 10 $nmol.kg^{-1}$ were highly toxic. When selenium was not included in the diet, the survival rate of the flies was about one-half that of the flies supplemented with optimal level of selenium (10 $nmol.kg^{-1}$).

Selenium in the environment can exist in various chemical forms. Selenate $[SeO_4]^{2-}$ (Se^{VI}) and selenite $[SeO_3]^{2-}$ (Se^{IV}) are the most common forms of selenium in the environment (14). Selenate is soluble and highly mobile in ground water and is the dominant inorganic form in neutral to alkaline oxidizing environments. Selenites are less soluble than selenates. Organic selenides may be produced when selenates and selenites are assimilated and reduced by primary producers (14).

Selenium also occurs as elemental form (Se^0), inorganic selenides (Se^{-II}) and a variety of organic forms (14).

Most organic selenium in animal tissues is present in two forms, selenomethionine which is incorporated in place of methionine in a variety of proteins, and selenocysteine, a cofactor for glutathione peroxidase, an enzyme of the antioxidant defense system, type I iodothyronine deiodinase and selenoprotein P (4). Glutathione peroxidase, uses glutathione to reduce peroxides in cells and, this way, protects membrane lipids and possibly proteins and nucleic acids from damage by oxidants and free radicals. Deficiency of type I iodothyronine may lead to hypothyroidism in the elderly. Selenoprotein P is an extracellular protein and may be involved in antioxidant functioning (4). Biomethylation of organic selenium compounds results in forms such as dimethylselenide (CH_3SeCH_3) and dimethyldiselenide ($CH_3Se_2CH_3$) which can be removed from the organism by volatilization. Precursors such as the trimethylselenonium cation $[(CH_3)_3\ Se]^+$ may occur during this process (27, 31). Table 1.1 summarizes some biologically relevant forms of selenium.

Table 1.1 Main selenium compounds found in biological samples

Name	Chemical formula
Selenate	SeO_4^{2-}
Selenite	SeO_3^{2-}
Selenomethionine	$H_3N^+ CH(COO^-)CH_2 CH_2 Se CH_3$
Seleno methyl selenocysteine	$H_3N^+CH(COO^-)CH_2\ CH_2 Se CH_3$
Selenocysteine	$H_3N^+ CH(COO^-)CH_2 Se^-$
Selenocystine	$H_3N^+ CH(COO^-)CH_2\ (Se)_2 CH_2(COO^-)CH\ H_3N^+$
Dimethylselenide	CH_3SeCH_3
Dimethyldiselenide	$CH_3Se_2 CH_3$
Trimethyl selenonium	$[(CH_3)_3 Se]^+$

1.4.2 Toxicity and Chemical form of Arsenic in Biology

Arsenic is an ubiquitous element present in air, water, soil and in living tissues. It is a metalloid that exhibits both metallic and nonmetallic properties. Elemental arsenic is a gray, crystalline material characterized by atomic number 33, atomic weight of 74.92 and chemical properties similar to those of phosphorus (14)

Arsenic compounds have long been recognized in terms of their poisonous characteristics. They have been used as homicidal and suicidal agents throughout history. Unlike selenium, arsenic has no confirmed essential requirement for humans or other animals. Arsenic compounds have been used in medicine since the time of Hippocrates, 400BCE. Arsenical drugs are used in treating certain tropical diseases, such as African sleeping sickness and amoebic dysentery, and in veterinary medicine to treat parasitic diseases. Arsenic trioxide looks promising in cancer therapy.

Arsenic in soil decreased plant growth (40) and killed vegetation (41). Arsenic has received significant public attention because of its link to certain types of cancers and its high levels in some drinking water supplies (42). Ingestion of large doses (70 to 180 mg) of arsenite is fatal to humans (4). Acute symptoms consist of fever and anorexia which may lead to cardiovascular failure. Other features include upper respiratory tract symptoms, peripheral neuropathy and gastrointestinal effects. Chronic exposure to inorganic arsenic compounds may lead to neurotoxicity, skin lesions and cancer. In contrast to most other human carcinogens, it has been difficult to confirm the carcinogenicity of arsenic in experimental animals (4). The mode of action of arsenic carcinogenicity has not been established**.**

Among the forms of arsenic in the environment, As^{III}, As^{V}, As^{0} and As^{-III} oxidation states are commonly available (14). Under oxidizing conditions or aerobic soils, inorganic arsenic is present predominantly in (+5) oxidation state mainly as arsenate. Under mildly reducing conditions, arsenic is primary present as arsenite, with arsenic in the +3 oxidation state. In arsenate, under highly basic conditions deprotonated arsenate exists as AsO_4^{3-}. At neutral pH arsenate exists as nearly equal mixture of $HAsO_4^{2-}$ and $H_2AsO_4^{-}$. At neutral pH arsenite exists mainly as $As(OH)_3^{2-}$ (43). In moderate reducing conditions, arsenic often combines with sulfur and iron to form insoluble sulfides (14). In strongly reducing environments, elemental arsenic or arsene, AsH_3 (-3) can exist (43). However, arsenic metal rarely occurs in nature. The forms of arsenic depend on the type and amount of sorbents, pH, redox potential and biological activity. Organic arsenicals such as arsenobetaine, arsenosugars and arsenic containing lipids are found naturally in many marine organisms. Table 1.2 summarzes some biologically relevant forms of arsenic.

Table 1.2 Main arsenic compounds found in biological samples

Name	Chemical formula
Arsenate	$HAsO_4^{2-}$ or $H_2AsO_4^-$
Arsenite	$As(OH)_3^{2-}$
Monomethylarsonic acid (V)	$CH_3AsO_3^{2-}$
Monomethylarsonic acid (III)	$CH_3AsO_2^{2-}$
Dimethylarsinic acid (V) [Cacodylic acid]	$(CH_3)_2As(OH)O$
Dimethylarsinic acid (III)	$(CH_3)_2As(OH)$
Arsenocholine	$(CH_3)_3AsCH_2CH_2OH$
Arsenobetaine	$(CH_3)_3AsCH_2CO_2$
Arsine	AsH_3

1.4.3 Importance of Interactions between Metals and Metalloids with Special Reference to Arsenic and Selenium

An organism may come in contact with more than one element at any given time. Interactions between mixtures of metals or metalloids can affect the absorption, biotransformation and excretion pathways of an organism differently from the isolated metal. In general, a number of terms are available to describe the interactions of chemicals in the environment (4). They are are summarized in Table 1.3. The effects of two chemicals given simultaneously may produce a response that may be *additive* of their individual responses. The effects are *synergistic* when combined effects are much greater than the sum of the effects of each agent given alone. *Potentiation* occurs when one chemical does not have a toxic effect, but when added to another element makes that chemical much more toxic. *Antagonism*

occurs when two chemicals are administered together and one decreases the action of the other. When present together, the joint action of selenium and arsenic may affect their speciation and interactions with biological systems and the toxicity may be expressed in different ways, including decrease or increase the toxicity.

Table 1.3 Interactions between metals and metalloids (4)

Effect	Response due to:		
	A in isolation	B in isolation	A + B together
Additive	2	3	5
Synergistic	2	2	20
Antagonism	4	6	8
Antagonism	4	0	1
Potentiation	0	2	10

Numbers (hypothetical) given here indicate the type of interactions between molecules A and B.

Toxicity to selenium and arsenic has been demonstrated in the field as well as in the laboratory. Arsenic in drinking water poses human health problems in many parts of the world including Bangladesh and chronic arsenic poisoning caused skin disorders followed by malignant tumors and eventually death in human populations (42). However, in some other parts of the world, high levels of arsenic in drinking water do not result in human health problems. The reason for this strange phenomenon may be attributed to antagonism between arsenic and selenium and resulting selenium deficiency in human populations affected by a

high level of arsenic. (44). The molecular basis of this antagonism was uncovered by X-ray Absorption Spectroscopy. Gailer et al. revealed antagonistic effects of selenite and arsenite in mammals with the formation of seleno-bis (S-glutathionyl) arsenium ion, $[(GS)_2AsSe]^-$ which may cause selenium defeciency in human (44). However, the existence of an equivalent detoxification mechanism in other animals is not known. A common weakness of this field of study is that, even though several metals and metalloids may present simultaneously, information on their joint effect on organisms is scant.

1.5 Research Objectives

Insects comprise a variety of life histories and, therefore, represent a good model for analyzing the effects of metals and metalloids in organisms. Insects have been useful in attempts to detect, measure and interpret environmental pollution by fertilizers, pesticides, nutrient enrichment, sewage and chemical waste disposal, radioactivity, mining and acid deposition (6). There is an increasing need to understand the environmental and trophic transfer of metals and metalloids by insects. However, speciation of metals and metalloids in insects has rarely been examined (27, 31) and therefore, very little information is available in this regard.

A significant problem concerning environmental impact of metals and metalloids is determining which form (species) of element is causing the toxicity and which form should be monitored. X-ray Absorption Spectroscopy (XAS) is an ideal technique to identify metal and metalloid speciation in biological samples with minimum pretreatment. Using XAS, Vickerman et al. studied the speciation of selenium in plant, insect and insect parasitoid association (31). When moving through different trophic levels, selenate biotransformed into organic selenium and produced precursers of volatile organic selenium indicating a possible mechanism of selenium elimination by insects. During the course of the present study, Freeman et al. reported the use of micro X-ray fluorescence imaging (μ-XRF) to show

selenium localization in a selenium tolerant moth feeding on selenium hyperaccumulating plant (45). These studies provided information on speciation of metals and metalloids in insects and their biological consequences using XAS. However, no information is known on the effects of speciation when more than one element is present.

In view of the large and very diverse group of animals that insects represent, this study attempts to understand selenium and arsenic speciation in different insects and to provide information for assessing environmental impacts of metals and metalloids.. X-ray Absorption Spectroscopy and micro X-ray fluorescence imaging techniques described in Chapter 2 were used to address the speciation and localization of selenium and arsenic in insects focussing on the following areas, which are included in Chapters 3-7 of this thesis.

Chapter 3: Speciation of selenium in insects inhabiting mining-impacted streams in Alberta, Canada.
Chapter 4: Characterization of selenium in insects adapted to feed on a selenium hyperaccumulating plant (*Astragalus bisulcatus).*
Chapter 5: Toxicity, biotransformation and localization of arsenic in bertha armyworm moth (*Mamestra configurata*).
Chapter 6: Toxicity,biotransformation and localization of selenium in bertha armyworm moth (*Mamestra configurata*).
Chapter 7: Effects of selenium and arsenic alone and in combination on the bertha armyworm (*Mamestra configurata*) and a wasp parasitoid (*Microplitis* mediator) of bertha armyworm.

Portions of the results of this thesis have been published in three peer-reviewed scientific journals (27, 30, 46). Prior to submission, manuscripts were reviewed by Dr. I. J. Pickering, Dr. G. N. George, Dr. H. Nichol and Dr. R. C. Prince.

Conclusions of this thesis and recommendations for future work are presented in Chapter 8.

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2. EXPERIMENTAL TECHNIQUES

2.1 Synchrotron X-ray Absorption Spectroscopy

X-ray Absorption Spectroscopy (XAS) is proving to be a unique *in situ* probe of metals or metalloids in biological materials. XAS uses an intense tunable source of X-rays generated by synchrotron radiation sources. XAS techniques have played an important role in finding answers to many questions related to chemical, biological and environmental science (1-4). Growing demand of this application is reflected at synchrotron radiation sources by an increasing number of beamlines devoted to XAS.

2.1.1 Synchrotron Radiation

In a present day synchrotron, when high-energy charged particles, such as electrons are traveling in a circular orbit at a speed close to the speed of light (relativistic electrons), they emit electromagnetic radiation tangential to the orbit and the radiation thus emitted is called synchrotron radiation (5). Electron accelerators that confine these relativistic electrons in the orbit are known as storage rings and synchrotron radiation sources. A typical storage ring comprises of symmetric straight and curved sections of stainless steel or aluminum tubes under ultra-high vacuum. Usually electrons are emitted from a hot tungsten (W) filament and are usually first accelerated with a linear accelerator and then with a booster ring of the synchrotron to the desired energy of the storage ring. The circumference of a storage ring is typically 50-1000 m and the electrons take about 0.15 to 3 μs to complete a circuit (6). Bending of these high-energy electrons either by the bending magnets or by the insertion devices such as wigglers and undulators results in emission of synchrotron beams.

These beams contain a majority of the electromagnetic spectrum ranging from the IR, visible, UV to the soft and hard X-ray regions. Of particular concern to most users are the brightness [number of photons per second per mm^2 (source size) per $mrad^2$ (angular divergence) per mA (current)] and flux (number of photons per second arriving at the specimen with certain bandwidth) of the storage ring at a given photon energy (6). The brightness is determined by the size and divergence of the electron beam and the flux is scaled to the ring current.

A beamline comprises three components: 1. the front-end which houses a safety valve and a photon shutter 2. beamline optics which involves apertures, slits, mirrors and monochromators 3. experimental stations at the end of the beamline. The most important device for all beamlines is the monochromator, which consists of two parallel single crystals of silicon (usually) that converts polychromatic synchrotron beam into a variable energy monochromatic beam. There are various monochromator materials available for a hard X-ray beamlines. For example, cutting the crystal along either the (111) or (220) planes give manageable angle for the energies of interest (4-40 KeV). Typically, optical elements such as mirrors of various surface figures are used to collimate and focus the beam both before and after the monochromator. In a modern XAS beamline, typically the upstream mirror collimates the beam through the monochromator and the downstream mirror focuses the beam onto the sample. Both mirrors also provide some harmonic rejection. Synchrotron radiation is extremely versatile for a large number of applications because of its brightness, tunability, polarization properties and coherence. The tunability of X-rays greatly facilitates techniques such as X-ray absorption spectroscopy and X-ray fluorescence imaging which will be discussed here in detail.

2.1.2 Features and Chemical Sensitivity of Near Edge Region and Extended X-ray Absorption Fine Structure

X-ray absorption spectra arise from core-level excitation by absorption of X-ray photons. During the XAS scan, low absorption occurs until the photon has energy close to that of the ionization energy of core electrons. At lower X-ray energies, X-ray-induced ionization of 2p or 2s electrons gives rise to L_{III}, L_{II} and L_{I} absorption edges. At significantly higher X-ray energies, ionization of 1s electrons give rise to the K X- ray absorption edge (Figure 2.1). A typical hard X-ray XAS beamline can provide an energy range between 5-25 KeV, giving access to K edges of elements through the second transition series and L edges for elements in the remainder of the periodic table (6). In an XAS experiment, the core hole created by absorption of an X-ray photon is filled by the decay of a higher level electron with concomitant emission of an Auger electron or a fluorescent X-ray photon. X-ray absorption can be monitored by measuring the transmission of X-rays through the sample, or by measuring the x-ray fluorescence, or the electron yield. In the hard X-ray energy range X-ray transmission is most useful for concentrated samples, while X-ray fluorescence has a much greater sensitivity and is useful for dilute compounds. XAS can be used to investigate solid, liquid, gaseous materials and any mixtures thereof (7).

X-ray absorption spectrum is usually separated into two different regions, the near edge region and the extended x-ray absorption fine structure (EXAFS) region (Figure 2.1). These two regions reflect different physical phenomena and will be discussed separately.

2.1.2.1 Near-edge Region

The near-edge region consists of features before the major inflection, and any features after the inflection which characterize the local electronic environment of the element (6). This region is also referred to as the X-ray absorption near-edge

spectrum (XANES), near edge X-ray absorption fine structure (NEXAFS) or edge region. Near-edge spectra are highly distinctive, and in many cases can be used to fingerprint an unknown chemical species by a comparison with spectra of known compounds. For example, elements with higher oxidation state have higher positive charge making it slightly more difficult to ionize the electron, shifting the K edge to a higher energy. Sensitivity of the near edge to determine oxidation state has been extensively used in biological and environmental research including research on speciation of selenium in insects (8), and in sediments (9), reduction of arsenic in indian mustard (3), arsenic and chromium biotransformation in desert plants (10, 11) and mercury speciation in fish (4).

2.1.2.2 X-ray Absorption Fine Structure

EXAFS measures the variation of the material's X-ray absorbance as a function of the incident energy, typically up to 1000eV or more beyond the absorption edge of a specific element (6). At energies higher than the absorption edge, oscillations are observed, which arise from interference effects resulting from the photoelectron wave ejected by the absorbing atom and the fraction of the photoelectron wave backscattered by atoms surrounding the absorbing atom.

Analysis of the EXAFS provides direct local structural information about the atom being probed. Sayers et al. were able to show that the Fourier Transform of an EXAFS spectrum gives peaks at distances corresponding to the radial distances of the excited atom to the neighboring coordination shells (12). This finding has lead to the development of methods to extract information on the number of neighboring atoms (coordination number), the identity of the neighboring atoms and their radial distances. Theoretical codes such as feff (13) have contributed substantially to EXAFS as a reliable structural tool.

Since the frequency of EXAFS oscillations are relatively easy to measure, interatomic (absorber-backscatterer) distances are measured quite accurately around

± 0.02Å. However, coordination number determination is less accurate (± 20-25%). This is caused by its correlation with the Debye-Waller factor (mean square relative displacement between the absorbing atom and the backscattering atoms), which is difficult to determine independently. In practice it is not possible to discriminate backscatterers with similar atomic numbers. For example, C, N and O are difficult to distinguish, as are P, Cl and S (6).

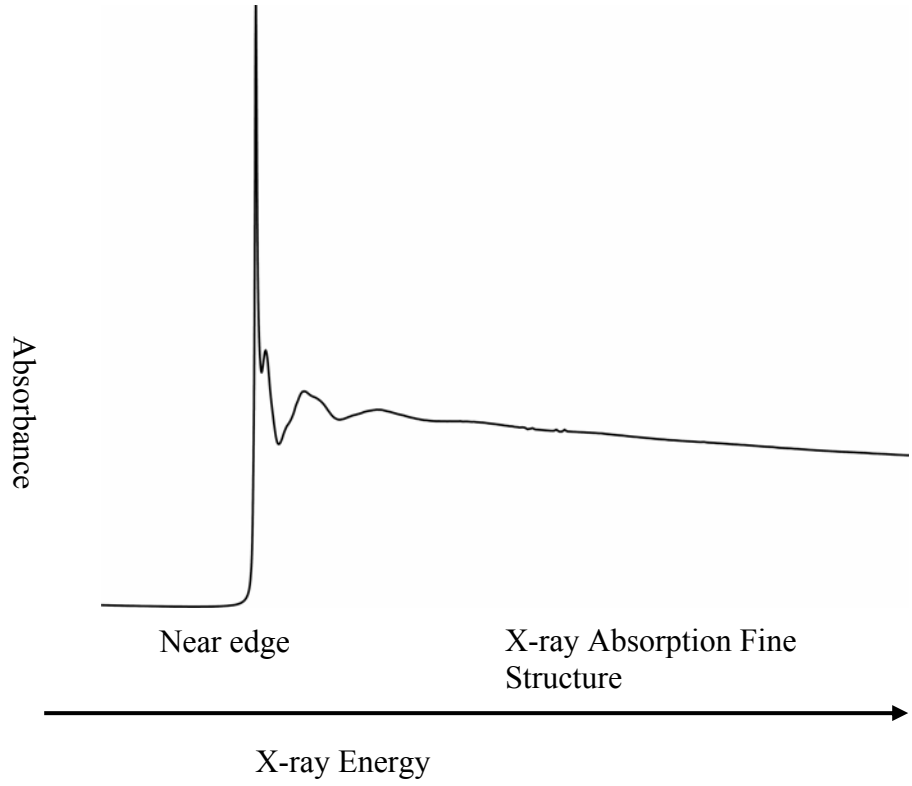


Figure 2.1 Features of X-ray absorption spectrum

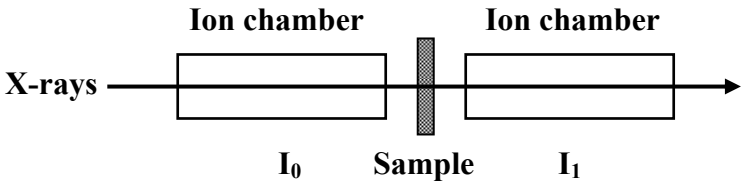
2.1.3 Fluorescence and Transmittance Experiments

The capability of XAS to measure gaseous, liquid or solid samples (including amorphous phases) is important. Due to penetration strength of X-rays, it is possible to examine a wide range of samples *in situ* in their natural state. X-ray absorption experiments can be done with different types of detection schemes, including transmission, fluorescence and electron yield. Transmission is used for concentrated samples, in which incident X-ray beam strikes the sample at 90° and the flux of the incident beam and transmitted beam is typically measured using a gas-filled ion chamber (X-rays ionize an inert gas and charged plates collect and record the microcurrent generated). A typical experimental set up for transmission is shown in Figure 2.2. However, in the transmittance mode, sample must be made impractically thin in order to measure the signal from concentrated samples. This problem can be overcome by mixing the sample with an inert substance like boron nitride.

Fluorescence is used for dilute samples, in which the beam strikes the sample at 45° and the fluorescent X-rays resulting from core electron excitations are measured using a gas-filled ion chamber or a solid state multi-element detector. For fluorescence detection, multi element Germanium detector is commonly used in experiments (14). In addition, X-ray filters are placed between the sample and the fluorescence detector to absorb unwanted fluorescence signal from other elements and scatter from the sample. Electron yield is used for enhanced surface sensitivity, in which the ejected electrons are measured using a metal grid or foil (6).

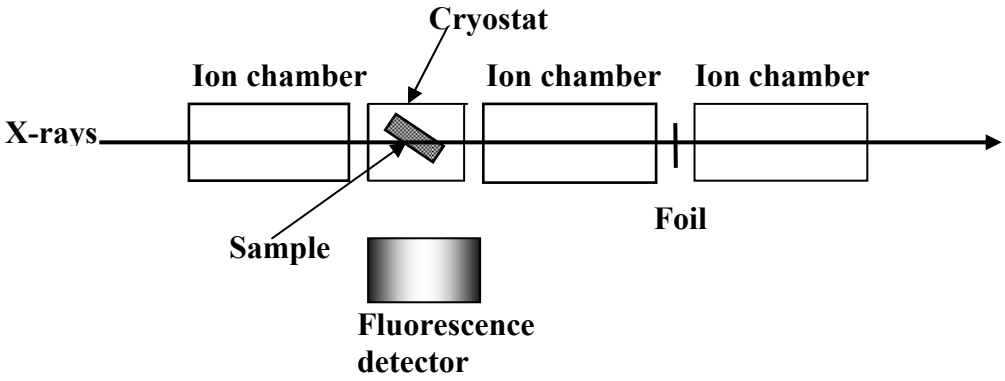
Keeping the sample at a low temperature is important to minimize the sample damage from exposure to intense X-ray beams and this is generally achieved by using a cryostat (Figures 2.3 and 2.4). Another advantage of using low temperatures is that low temperatures minimize thermal vibrations and maximize the EXAFS amplitudes.

Ice diffraction causes intense diffracted beams to strike the detector, which yield diffraction peaks in the spectra. Adding glassing agents such as glycerol or flash freezing in a mixture of isopentane and liquid nitrogen can remedy this.



Absorbance (A) = log (I_0/I_1)

Figure 2.2 Schematic of a typical X-ray absorption spectroscopy transmission experiment



Absorbance (A) α (I_f/I_0)

Figure 2.3 Schematic of a typical X-ray absorption spectroscopy fluorescence experiment

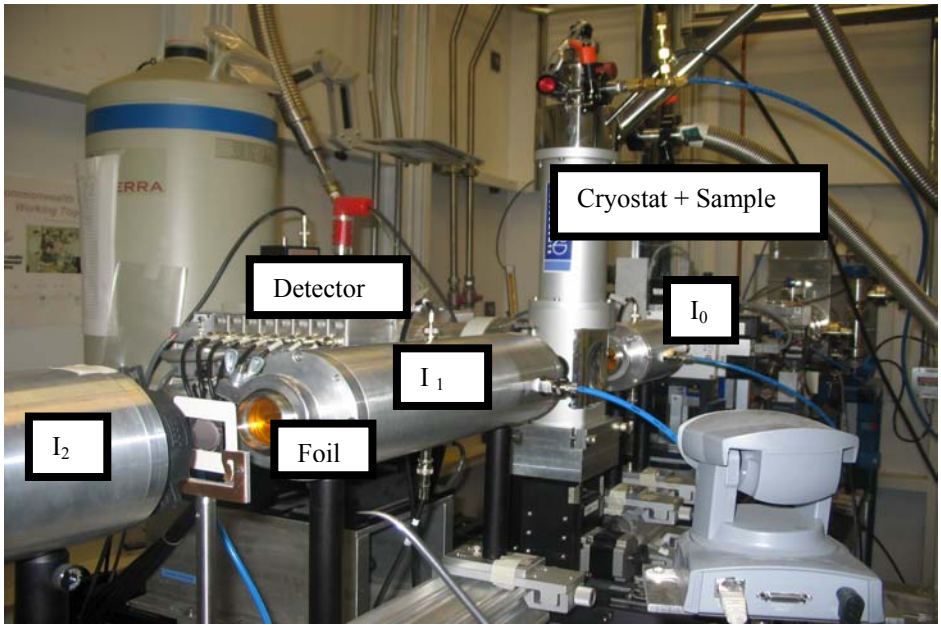


Figure 2.4 X-ray absorption spectroscopy set up on the Hard X-ray Microanalysis (HXMA) beamline at the Canadian Light Source (CLS) showing sample containing cryostat, detector, foil and ion chambers (I_0, I_1, I_2)

2.1.4 Cautions for X-ray Absorption Spectroscopy

2.1.4.1 Beam Damage

Intense X-ray beams can cause damage to the samples. In organic materials, radiation damage has been shown to cause breaking of disulphide bonds, removal

of hydroxyl groups and decarboxylation of certain acids (15, 16). Radiation damage can be minimized by cryocooling the sample in order to decrease the mobility of the free radicals (mainly electrons) formed during the irradiation.

Photoreduction and photooxidation of the target element are also causes for concern. Organic molecules in the samples, material used in sample preparation are sources of electrons for reduction of metals and metalloids in the sample. For example, reduction of selenate to selenite can occur during exposure to the synchrotron beam or even at cryogenic temperatures. It is important to take multiple sweeps of data and compare the near edges over time to check the data for photoreduction and photooxidation. Faster sweeps, lower temperatures and exposing a new spot of the sample by moving the beam can remedy this.

2.1.4.2 Problems with Sample Preparation

The effect known as pinhole effect is seen when doing a transmission spectrum in an inhomogeneous sample. In this case, most of the beam goes through the gaps or thin parts of the sample distorting the signal. Therefore, it is important that all of the elements being probed exist in a homogeneous type of state by making a uniform sample. Thickness effect or self absorption effect occurs when fluorescence data is collected on samples which are too concentrated.

2.2 Synchrotron X-ray Fluorescence Imaging Techniques

The ability to view and record biological structures and biochemical processes within them enhance our understanding on anatomy, physiology, biochemistry and evolution. In general, synchrotron imaging is useful for materials in which the element is in the dilute limit, and to materials which are thin enough to penetrate the sample without substantial attenuation (17). This technique can examine biological materials ranging from subcellular components through cells and intact

tissues to whole organisms (7). As the image is built up pixel by pixel, the size of the pixel depends on the size of the sample and the details to be addressed. Typically, these experiments are done on a focused beam in which the beam is made smaller using apertures, zone plates, tapered capillaries and Kirkpatrick-Baez (K-B) mirrors. They are capable of providing spatial resolution of ≤ 1μm (6,18). A sample manipulation stage is required to position the sample in the X-ray beam and also to move the sample along X and Y coordinates. An optical microscope is used to view sample/beam position. Typically, the entire set up is installed on a stage driven by a lift table, which allows precise positioning of the set up with respect to the incoming beam (Figures 2.5 and 2.6).

2.2.1 Microprobe or Micro X-ray Fluorescence Imaging or μ-XRF

Taking the advantage of increasing brightness of X-ray sources and the use of focusing optics it is possible to locate trace elements within the heterogeneous matrix (gases, liquids or solids) in biological and environmental samples. For this, spatially resolved elemental mapping is obtained by moving the sample point by point (raster scan) in the X-ray beam using a fixed energy above the excitation energy of the elements of interest. Following collection of the image, micro X-ray absorption spectra (μ-XAS) can be collected at selected pixels, chosen based on the X-ray and/or optical map. These spectra provide detailed information at those particular points. Since it is time consuming to collect spectra for each pixel in the entire image, taking data at selected pixels may miss important areas of the sample.

2.2.2 XAS Imaging/Chemically Specific Imaging/ Oxidation State Imaging

If a sample has two or more chemical species, XAS imaging can be used to generate maps of these chemical species. It requires pre knowledge of the chemical forms in the sample from bulk spectroscopy and works best for mapping species

with large contrast in edge. This technique involves raster scanning the sample multiple times. At first, energy is moved to one edge energy and then raster scan the sample horizontally to collect fluorescence images. This procedure may be repeated with different edge energies. It is preferable to collect all the energies for one horizontal raster in a short amount of time before moving to the next raster. During imaging, beam damage can be minimized by reducing the irradiation duration and time between the start and completion of data collection for each pixel. XAS imaging will provide insights into whether the different chemical forms of the element are diffusely distributed or specifically localized in special cells, or organs. Information about their distribution and co-localization with other elements can also be obtained.

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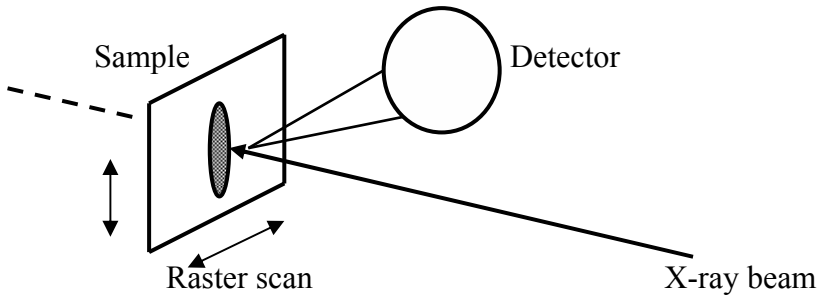

Figure 2.5 Schematics of Micro X-ray fluorescence imaging set up

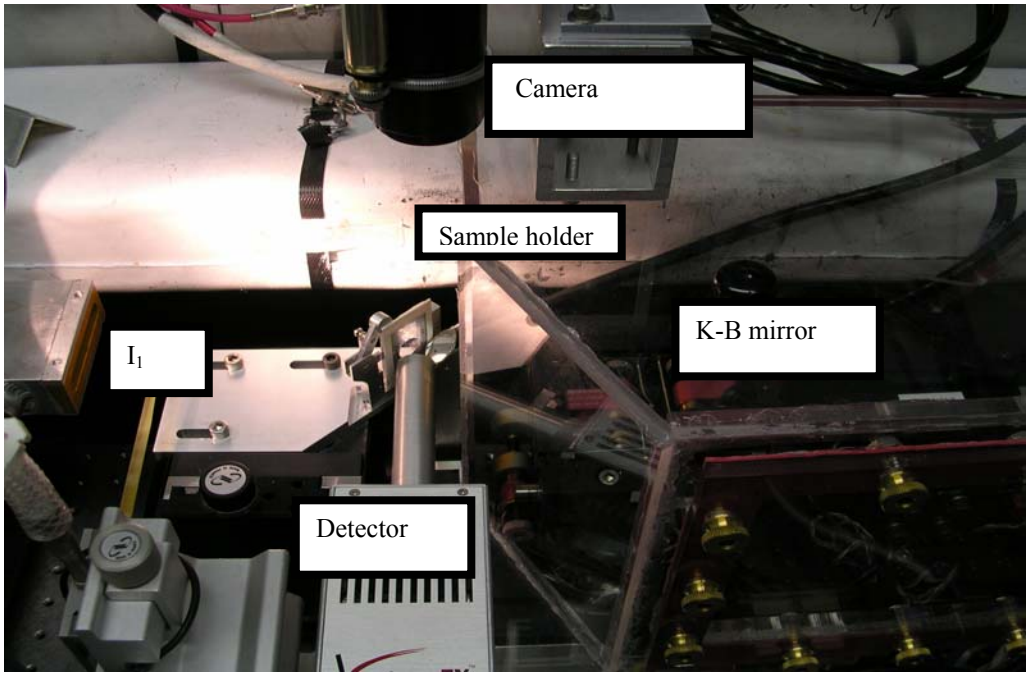

Figure 2.6 Micro X-ray fluorescence imaging setup on beamline 7-3 at the Stanford Synchrotron Radiation Light Source (SSRL) showing ion chamber (I_1), sample holder, K-B mirror, camera and detector

2.3 Advantages and Disadvantages of X-ray Absorption Spectroscopy

Synchrotron-based X-ray Absorption Spectroscopy is a powerful tool that can be used to identify the chemical species of an element in complex environmental samples. It can be applied to samples as different as sediments, biofilm and intact tissues. It requires only minimal pretreatment, and can determine the chemical form in the entire element present in the sample, whether precipitated, aqueous dissolved, or in any other form. However, this technique only provides information about the chemical types and cannot be used to identify specific molecules. Another disadvantage is that this technique will not detect minor components of the element in the presence of a large excess of other components.

2.4 Experimental Facilities

Experiments were carried out at two third generation synchrotron facilities: Stanford Synchrotron Radiation Lightsource (SSRL) and Canadian Light Source (CLS). The SSRL is a part of the Stanford Linear Accelerator Center (SLAC). Presently, X-rays produced by Stanford Positron Electron Asymmetric Ring has a capacity of 3 GeV energy (SPEAR 3) and 200 mA ring current. SSRL currently has eight beamlines that are suitable for hard X-ray absorption spectroscopy. The CLS uses electrons from Linear Accelerator Centre (linac) and is now generating 2.9 GeV energy with 250 mA ring current.

2.5 Spectroscopy of Bulk Samples

Bulk tissues were examined on beamlines 9-3 at Stanford Synchrotron Radiation Laboratory and Hard X-ray Microanalysis (HXMA) beamline at the Canadian Light Source (CLS). These beamlines were equipped with liquid helium cryostats to keep the sample at low temperature. Samples were kept frozen at 10K during data collection. X-ray fluorescence was detected by 30-element Germanium detector or Stern-Heald-Lytle detector. Experimental setup and data collection were carried out by XAS collect data acquisition program.

2.5.1 Analyses of Near-edge Spectra

2.4.1.1 Background Subtraction and Normalization

Background subtraction, normalization and data analyses of XAS data are carried out by computer programs, a range of which are available. The EXAFSPAK program suite (19) was used throughout this work. The first few steps of the analysis are basically the same for near edge and EXAFS.

A data file contains all detector channels of a single scan. Before processing, the individual scans are visually inspected for quality and consistency

of the channels. If a channel seems to be contributing excess noise compared to others, it should be excluded from the analysis. Energy scales of the sample spectra are then calibrated with respect to the absorption features of a reference spectrum. Since X-rays detected are dependent on the source and optic settings, it is important to collect the calibration spectrum under similar conditions to the sample spectra on a calibrated beamline. Then the data files are averaged into a single average file.

The next step is to remove the pre-edge background to isolate the contribution from only the element and edge of interest. The pre-edge background signal obtained on transmission mode includes contribution from lower energy absorption edges of the other elements in the sample and from gases in the ion chamber. Fluorescence background signal includes fluorescence from lower absorption edges and from elastic and inelastic scatter. Background subtraction involves fitting the pre edge with a function of energy and then extrapolating the function into the post edge region and subtracting it from the data. Background subtracted spectra are characterized by flat and zero pre edge and a smoothly decreasing post edge.

2.4.1.2 Principal Component Analysis of Near Edge Spectra

Principal component analysis (PCA) has been used to gain insights into near edge spectra of samples containing a mixture of chemical compounds (20). It provides a statistical basis for choosing the number of standards to include in subsequent analyses. A set of spectra from samples which have related compositions are analyzed to provide information on the minimum number of components in that set of spectra. Data need to be normalized and background subtracted to obtain a common energy scale for all spectra. The experimental data is first mathematically converted to a data matrix and is devoid of any physical or chemical meaning.

PCA is used to determine the minimum number of significant components required to satisfactorily regenerate the data matrix. The most significant eigenvalues reveal eigenvectors that are considered as true sources of structural variation in the data matrix and constitute the primary set of *n* eigenvalues. The smallest eigenvalues and their eigenvectors are left out in the reproduction of the data matrix and are considered as experimental error. The *n* primary eigenvectors indicate the presence of *n* distinct components in the original spectral mixtures.

2.4.1.3 Target Transformation and Least Square Fitting

Significant components n obtained from PCA, represent the main independent sources of variation in the experimental data, but have no chemical or physical meaning. Target transformation is a subsequent step that allows testing of suspected targets such as chemical species used as standards. The standard is accepted, if the spectral signature can be successfully reconstructed using the eigenvectors retained from PCA. Target transformation ranks and identifies the most likely standards, so that the fitting (least square fitting of near Edges or DATFIT) can be done on a smaller set of standards. Using least square fitting, it is possible to determine the relative content of different chemical species in the sample by fitting the sample spectra to the spectra of standard species selected from the target transformation. An example plot of least square fitting is given in Figure 2.7. Based on the experiment in Chapter 3, this was obtained by fitting the caddisfly spectrum in Figure 3.5 to the standard spectra given in Figure 3.6. In Figure 2.7, caddisfly spectra and fit were plotted with standard spectra according to their contributions to the sample. Conclusions that can be drawn from this analysis include the elemental speciation and composition of different chemical species in the sample.

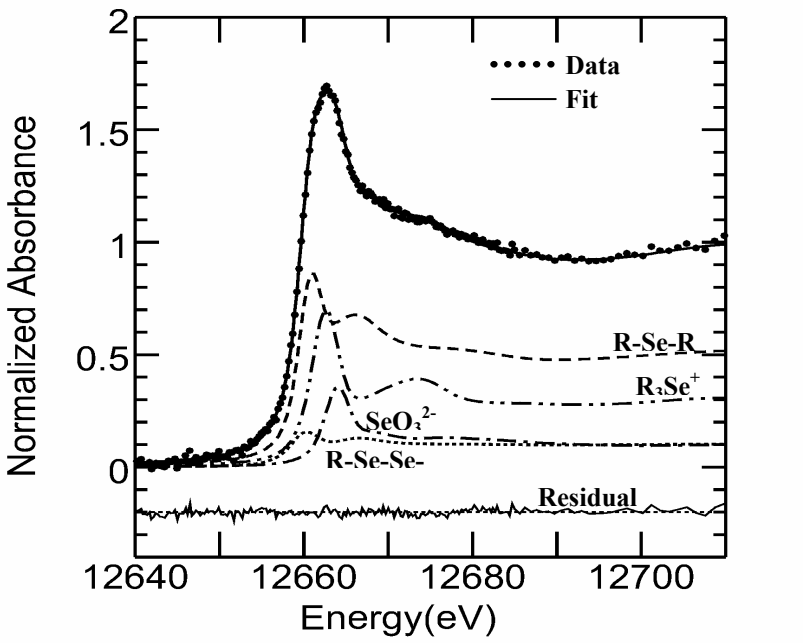

Figure 2.7 Least square fit of the spectrum of caddisfly (*Rhyacophila* sp.) pupa described in Chapter 3. Near edge spectra of this insect (Figure 3.4) was fitted to the sum of spectra of standard selenium species (Figure 3.5). Figure shows the caddisfly data, the fit and the individual components, scaled according to their contributions to the fit: SeO_3^{2-} (selenite), R-Se-Se-R (diselenides), R-Se-R (selenides), R_3Se^+ (trimethylselenonium). The residual is shown offset below

2.5.2 Analysis of X-ray Absorption Fine Structure (EXAFS)

In EXAFS analysis, a spline is fitted through the oscillatory part of the data removing the lowest frequency oscillations without affecting the higher frequency EXAFS. Then EXAFS is extracted as $\chi(E)$ by calculating spline and scaled data and interpreted as $\chi(E)$ *vs* energy (E). EXAFS oscillations are now clustered to low E. The next step is to convert them to uniform k space (wave vector) and then weigh to high k. To avoid underestimating the value of EXAFS at high k, it is common to multiply the raw EXAFS by a weighting function such as k^3 to

emphasize the high k oscillations. A Fourier transform of the k^3- weighted EXAFS is useful to visualize shells at different distances. Peaks in the Fourier transform give approximate radial structure, which can be used as a starting point in curve fitting to derive structural information from the EXAFS. Fourier transforms can be phase corrected for the first shell to get more accurate radial distances. Extraction of the structural information utilizes single or multiple scattering theory to simulate the EXAFS (13). The procedure is to propose a starting model for the structure, simulate the EXAFS, and then vary the parameters such as number of atoms and inter-atomic distances to obtain the best fit. Other information such as chemical suitability for the choice of ligands, interatomic distances and coordination numbers can also be considered. For a reasonable fit, the Debye-Waller factor has to be in a reasonable range between 0.0015 $Å^2$ and 0.0080 $Å^2$. EXAFS signal $\chi(E)$ can be mathematically expressed as follows.

EXAFS Equation:

$$\chi(k) = S_0^2 \sum_i \frac{N_i A_i(k)}{kR_i^2} e^{-2R_i/\lambda_i(k)} e^{-2\sigma_i^2 k^2} \sin\left[2kR_i + \phi_i(k)\right]$$

N_i - Coordination number of atom i

R_i - absorber-backscatterer distance from atom i

σ_i^2 - mean-square deviation in R_i (Debye-Waller factor)

S_0^2 - total amplitude reduction factor

$\lambda(k)$ -photoelectron mean free path function

$A_i(k)$ - total EXAFS amplitude function

$\phi_i(k)$ - total EXAFS phase shift function

k - photoelectron wave vector

2.6 Micro X-ray Fluorescence Imaging (μ-XRF) and X-ray Absorption Spectroscopy (XAS) Imaging of Insect Larva

X-ray fluorescence imaging (μ-XRF) and X-ray absorption spectroscopy (XAS) imaging were used to image insect larvae treated with 100 μm of arsenate and 100 μm of selenate in two separate experiments (Chapter 5 and 6). This technique has been previously described by Pickering and coworkers (21, 22), and was designed to give complete structural information in a dilute sample with very low irradiation time. In μ-XRF, each insect was rastered in the beam using energies well above the absorption edge of the element under study (selenium and arsenic). Energies for XAS imaging were based on the chemical forms in insects obtained from bulk spectroscopy and the chemical form in the diet. Two energies used for arsenic imaging were corresponding to the X-ray absorption of standard solutions of arsenate at 11874.9 eV (chemical form in the diet) and As^{III}-*tris*-glutathione at 11869.7 eV the main chemical form found in insects.

Experiments were carried out on beamline 9-3 at Stanford Synchrotron Radiation Lightsource, where microfocus beams were provided by a tapered glass monocapillary optic (X-ray Optical Systems, Inc, East Greenbush, NY, USA). This capillary does not require adjustment along the beam direction on changing energy so that the images taken at different energies are spatially aligned (23). A 10-μm diameter beam spot was achieved at the focal point of the optic and the sample was moved downstream to a slightly defocused position to achieve 20 and 40 μm diameter spots. The spot sizes were characterized by scanning a knife edge through the beam. The beamline energy was calibrated with reference to an arsenic metal foil before each image was recorded.

Each bertha armyworm larva was mounted live, and was positioned vertically in a thin-walled quartz capillary at room temperature. The larva was immobilised by flowing nitrogen gas through the capillary, which acted as an anaesthetic; offline tests showed that larvae could survive many hours under these

conditions. The larva was mounted on a mechanical stage (Newport Corporation, Irvine, CA, USA) fitted to a computer-controlled micro stepping motor that move the sample in the *x, y* position for raster scanning. Sample was centered to the incident beam allowing the sample to be visualized with an optical microscope and a camera. Arsenic Kα fluorescence and scatter intensities were monitored using a 50-mm silicon drift detector (Vortex SII Radiant Technologies, Northridge, CA, USA), mounted upstream of the sample at approximately 55º to the incident beam.

Energies for the scan were based on the speciation results obtained from bulk XAS measurements. A horizontal raster scan was collected at energies of the chemical forms of arsenate at 11874.9 eV (chemical form in the diet) and As^{III}-*tris*-glutathione at 11869.7 eV (major chemical form in insects) and then the sample was moved vertically, thus ensuring that data were collected on a given pixel in the shortest amount of time (7).

For selenium treated larvae, the selenium chemical forms used in XAS imaging were, selenate, which is the chemical form in the diet (12667 eV) and R-Se-R (modeled by selenomethionine) which is the major chemical form in the insect (12661 eV). Energy was calibrated by using the spectrum of hexagonal Se (0) and the first inflection point was assumed to be at 12658 eV. The distributions of other metals such as copper and zinc were also monitored simultaneously with those of arsenic in arsenate treatment, and selenium in selenate treatment to observe possible co-association of these metals with arsenic.

Data were collected using the SCAN_CONTROL software, which consists of two programs that run concurrently, X Windows graphical user interface (GUI) and data acquisition program. These two programs have data manipulation, analysis and display capabilities.

2.6.1 Imaging Data Analysis

Total elemental distribution was mapped using Sam's microprobe analysis kit (SMAK) (24). Further to this, quantitative concentration maps can be obtained by calculating fluorescence intensities of the images, intensity of the corresponding model compound and concentration of the model compound using the methods described by Pickering and George (7). This analysis relies on the approximation that the sample consists of 100% water (7).

2.6.1.1 Determination of molar amounts of each component

The first step of the analysis should be to remove the contribution of scatter or fluorescence from other elements from the fluorescence signal. Since energy for XAS imaging is lower than the energy for μ-XRF, elastic scatter from XAS imaging is lower in energy and may overlap with the fluorescent signal. Therefore, it is important to remove scatter from windows counts.

For a given energy, more than one component may be contributing to the fluorescence map. Also, the thickness of the sample may not be uniform throughout the sample. Therefore during data processing, the amount (molar quantities) of each chemical species is first calculated from the corrected fluorescence intensities using the equation (1). Then the effective thickness of the sample can be calculated using absorbance measurements (equation 2). Elemental concentration is calculated by dividing the molar quantities from the effective thickness and pixel area (3). This technique provides complete maps of chemical forms in the sample. However, in this study, the larva was mounted in a quartz capillary and therefore, it was not possible to calculate the thickness to obtain the concentration of the chemical forms.

.

$$F_j = k_q \sum_i m_i I_{ij} \quad (1)$$

F_j - Fluorescence correspond the *j* energies of observation

I_{ij} - Normalized intensity of the standard spectrum of component *i* at energy *j*.

k_q - Quantitation standard, which can be measured from a standard of a known concentration.

In this $J_s = k_q m_s$

where *J* is the observed height of the edge jump for a standard *s and* m_s is the moles per pixel for the standard. For a standard of known concentration c_s and t_s

$$k_q = J_s / c_s a t_s$$

2.6.1.2 Calculating Effective thickness

Effective thickness per pixel (*t*) can be calculated using the following equation:

$$t = \frac{(A - B)}{\sigma \rho} \quad (2)$$

where *A* is measured absorption per pixel which consists of a absorption from windows, gases etc**.** *B* is the absorption without the sample. σ is the absorption cross section and ρ is the density both assumed to be that of water.

2.6.1.3 Deriving Concentrations

Concentrations c_i can then be calculated from the molar amounts m_i and the volume (*at)* as follows.

$$c_i = \frac{m_i}{at} \quad (3)$$

2.7 Atomic Absorption Spectroscopy

Atomic absorption spectroscopy (AAS) is frequently used for quantitative analyses of elements in many laboratories (25). The basis of AAS is the absorption of discrete wavelengths of light by ground state, gas phase free atoms. The hydride generation (HGAAS) technique uses a quartz cell, which is heated by a flame or electricity. Elements in the sample react with sodium borohydride in a closed reaction system external to the spectrometer and form volatile hydride compounds. Arsenic, for example, forms arsine gas, AsH_3. Hydride species are then reduced to free atoms in the heated quartz cell and the absorption signal is measured.

Calibration curves are prepared from solutions of known concentrations of the sample element. First, a stock solution is made from the element of interest and then it is diluted to make a series of working standards. The concentration of the working standards must cover the complete range of concentrations of the samples to be determined (three to five working standards are typical). A calibration blank is required to correct any analyte present in the water or reagents used to prepare the standard solutions. A method blank is also required using the same sample preparation steps as the sample. The method blank is used to correct for any contamination that may have occurred during sample preparation. These standard solutions are aspirated into the flame and the absorbance of each standard is measured and the absorbance is plotted against concentration. The concentration of the unknown is determined from the calibration curve. It should be noted that the relationship between absorbance and concentration is linear over a certain range, but at higher concentrations the relationship deviates from linearity. Provided that the slope of the curve is fairly steep, it may be possible to distinguish

concentrations outside the linear range. Above a certain concentration level, the curve becomes so flat that accurate concentrations are difficult or impossible to obtain. During sample analysis, the sample is atomized and the absorbance is measured under exactly the same conditions as the calibration standards were measured. The absorbance of the method blank is subtracted from the sample absorbance to give the correct sample absorbance.

In atomic absorption spectroscopy, analytes in solid samples must be released from sample matrix before determination. To prepare the samples in liquid forms, samples are digested with nitric acid or hydrogen peroxide. Also, the efficiency of hydride formation depends on the oxidation state of the analyte. In the case of selenium, calibration standards and selenium containing samples must be in the Se (IV) form and for analysis of arsenic, the standards and the samples must be in the As (III) form.

Sample Preparation and Atomic Absorption Spectroscopy Analysis

Samples were prepared for analysis following procedures used by the Alberta Research Council (26). Samples were oven dried, and 0.5 g of dried sample was microwave digested in 5 ml of nitric acid. Digested solutions were then diluted to 100 ml using deionized water. Total elemental concentration was measured using hydride generation atomic absorption spectrometry (Varian AA240) in the Department of Soil Science, University of Saskatchewan. Five standard solutions of selenium and five arsenic solutions ranging from 1 to 20 $\mu g.l^{-1}$ were used for selenium and arsenic analysis respectively. Blank samples including reagent blanks and digestion blanks were analyzed together with insect samples. Two independent measurements were made of each sample.

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3. SPECIATION OF SELENIUM IN INSECTS INHABITING COAL MINE-IMPACTED STREAMS NEAR HINTON, ALBERTA

3.1 Introduction

Selenium contamination in the environment has received considerable attention from the general public, scientists and governments in various parts of the world. At elevated levels, selenium is toxic to fish and wildlife and exhibits a high potetntial for bioaccumulation through food chains (1, 2). While some areas of North America are relatively low in selenium, the levels in other areas are sufficiently high to be of concern. Selenium is released into the environment from a variety of human activities including mining, agriculture, industry and burning of coal and other fossil fuels (2).

Selenium from naturally seleniferous soils can leach through agricultural irrigation and rainfall and accumulate in evaporation ponds leading to toxicity in birds and fish (3, 4). For example, at the Kesterson National Wildlife Refuge in California, irrigation water draining seleniferous soils in the San Joachim Valley was added to Kesterson wetland ponds. High selenium levels caused deformity and death of adult and hatchling aquatic birds, including ducks, slits, grebes and coots and the elimination of most species of fish (3, 4). Southwestern Saskatchewan and southeastern Alberta in Canada likewise contain geologically seleniferous areas with an arid climate and closed drainage basins. These areas usually require irrigation for agricultural crop production, which may lead to selenium contamination in subsurface areas and increased risk to the food chain organisms (5). Recent studies have revealed elevated levels of selenium in fish tissues and larval deformities in fish downstream of some uranium mines in northern Saskatchewan (6).

Near Hinton, Alberta, coal-mining activities have increased metal and metalloid levels in surrounding streams raising questions about potential risks associated with exposure to elevated levels of selenium. Coal has been mined in the Rocky Mountains of Alberta and British Columbia since the late 19th century (7). At these open-pit mines (Figure 3.1) waste materials are generally deposited on the surface in tailings piles, ponds, landfills and dumps. Surface water may drain from these deposits into nearby aquatic habitats and eventually streams that drain the local watershed (8).

Dietary exposure is considered be the dominant pathway by which selenium enters into animals (9*,* 9), and Wayland et al. found levels of selenium in insects living in Luscar Creek and Gregg River near Hinton that were sufficiently elevated to be a potential risk to insectivorous birds and fish (10,11). Insects such as mayflies, caddisflies, stoneflies, and craneflies inhabit these streams. They serve as primary consumers (feeding on plant material), predators (feeding on other animals), and detritivores (feeding on dead plant and animal matter), and are themselves consumed by animals at other trophic levels such as other insects, fish, amphibians and birds. These include insectivorous fish such as trout, and birds such as american dipper (*Cinclus mexicanus*) (Figure 3.2) and harlequin duck (*Histrionicus histrionicus*).

Figure 3.1 Coal mining activities near Hinton, Alberta

Figure 3.2 An american dipper (*Cinclus mexicanus*) [circled in yellow] feeding on underwater insects in the Gregg River stream

Understanding and predicting the impact of elevated selenium levels on biota require knowledge not only of the total selenium levels but also of the selenium species present in the various trophic levels. The chemical form of selenium present substantially governs selenium bioavailability and toxicity.

Possible mechanisms in which selenium may cause toxicity in birds include: i) Formation of methylated selenium which either enters a redox cycle and generates superoxide and oxidative stress, or forms free radicals that bind to and inhibit important enzymes and proteins (13, 14), ii) Production of excess selenocysteine and inhibition of selenium methylation, which causes hepatotoxic and other adverse effects (13), iii) Formation of excess selenium analogs of sulfur-containing enzymes and proteins (14, 15). The mechanism underlying the toxicity of selenium in fish is assumed to be the substitution of selenium for sulfur during protein synthesis (16), but detailed mechanisms of toxicity in fish are currently unclear.

Traditionally, chemical forms of selenium are identified using techniques requiring sample pretreatments such as digestion that may alter the chemical form. Selenium K-edge XAS is sensitive to chemical form (17) and has previously been used for analysis of organic and inorganic selenium in environmental samples (18), including insects (19, 20). In this study, we use XAS to identify the chemical forms of selenium in insects from mine-impacted streams near Hinton, Alberta.

3.2 Materials and Methods

3.2.1 Study Site

Samples were collected from two coal mine-impacted streams, Luscar Creek (53° 3' 25" N, 117° 19' 58" W) and Gregg River (53° 5' 18.4" N, 117° 26' 17.9"W) during the last week of May 2005. Samples were collected no further than 15 km downstream from the coalmines. In these streams, the concentration of selenium in the water ranged from 1.3 to 9.2 $\mu g.l^{-1}$, which is higher than the Canadian Council of Ministers of the Environmental Guideline of 1 $\mu g.l^{-1}$ (10).

3.2.2 Field Sampling

An aquatic net of 500 μm mesh was used to collect random insect samples from approximately 100 m length of stream at each sampling site. Insects were collected

by kick sampling, in which the net is held underwater with the opening facing upstream and the substrate in front of the net is disturbed by kicking, rubbing and tumbling. This process dislodges the organisms that are residing among the various substrates and the current carries them into the net. Each kick sample was sorted into families and genera (Table 3.1) based on the keys and descriptions from Merritt and Cummins (20). In order to keep the insects alive during this sorting process, they were placed in a container that kept them in constantly flowing stream water (Figure 3.3). Kick sampling was repeated at random locations in the stream, and insect samples were pooled until sufficient numbers of insects from each taxa were collected. Approximately sixty mayflies, and the equivalent volume of other insects, were collected.

Abundant genera and those representing a wide range of feeding habits were chosen for analyses (Table 3.1). The immature stages of insects belonging to the orders Ephemeroptera (mayflies), Plecoptera (stoneflies), Trichoptera (caddisflies) and Diptera (craneflies) were sampled. Samples included nymphal stages, larval stages and pupae of *Rhyacophila* sp. as well as exuvia (molted skins) of stoneflies. Larvae of the genus *Tipula* are poorly understood taxonomically and contain both detritivorous and predaceous cranefly species (20); although further identification of this larva was not possible, the most common cranefly species found in Alberta streams are detritivores. Biofilm samples (mainly periphytic algae, also bacteria, fungi, invertebrates and sediments) from the rock surfaces (five randomly selected rocks per site) were additionally collected. A portion of each insect and biofilm sample was rinsed in distilled water and rapidly frozen in liquid nitrogen at the field site to preserve the selenium chemical form for XAS analysis, and the rest was preserved on dry ice.

Table 3.1 Insects collected from Luscar Creek and Greg River and used in the analyses

Order (Common name)	Family	Species	Feeding habit
Ephemeroptera (mayfly)	Ephemerellidae	*Epeorus* sp.	Herbivore
Ephemeroptera (mayfly)	Ephemerellidae	*Drunella* sp.	Herbivore
Ephemeroptera (mayfly)	Heptageniidae	*Rhithrogena* sp.	Herbivore
Trichoptera (caddisfly)	Rhyacophilidae	*Rhyacophila* sp.	Predator
Trichoptera (caddisfly)	Hydropsychidae	*Parapsyche* sp.	Omnivore
Plecoptera (stonefly)	Perlodidae	*Megarcys* sp.	Predator
Diptera (cranefly)	Tipulidae	*Tipula* sp.	Detritivore/Predator[a]

[a]Larvae of the family Tipulidae are poorly understood taxonomically, and therefore their feeding habits could only be described as detritivorous or predaceous

Figure 3.3 Insects were kept in flowing water during sorting process

3.2.3 Total Selenium Analysis

Insects and biofilm samples were prepared for total selenium analysis as described in Chapter 2 and references therein. Fifty mayflies per sample, and for other insects a sample equivalent to that volume of mayflies, were used in the analysis. Two independent measurements were made of each sample.

3.2.4 X-ray Absorption Spectroscopy

Frozen insects, 5-10 insects per sample, were ground in liquid nitrogen, packed into 2 mm-pathlength sample cells and kept frozen in liquid nitrogen for XAS analysis. XAS data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 9-3 as described in Chapter 2. Spectra of dilute aqueous solutions of standard selenium species, buffered at pH 7 were also collected. These included the inorganic selenium forms selenate (Se^{VI}) and selenite (Se^{IV}), the organic forms selenomethionine, trimethylselenonium and selenocystine as well as solid elemental selenium (recorded in transmittance). Background subtraction, normalization and data analysis were carried out according to the methods described in Chapter 2.

3.3 Results and Discussion

3.3.1 Total Selenium Level

Total selenium content showed variations between the two sampling sites and among the insect taxa (Figure 3.4). Samples from Luscar Creek consistently had slightly higher average levels of selenium than the samples from Gregg River. These results are in accordance with an earlier study that showed Luscar Creek as the most contaminated stream in the study area (21). In the current study, biofilm samples had lower selenium levels than insects.

Selenium is an essential micronutrient in animals. Trace amounts of selenium are required for normal growth and development. At moderate levels, storage and body homeostasis are maintained. High levels of selenium can cause toxicity. Previous data on dietary toxicity thresholds of selenium for fish and birds ranged from 3-11 $\mu g.g^{-1}$ dry weight with the majority of threshold values being ≤ 7 $\mu g.g^{-1}$ dry weight (9). Selenium levels in all insects in our study (> 4 $\mu g.g^{-1}$ dry weight) exceeded the lower threshold toxicity limit for fish and birds. Two of the

Luscar Creek samples, *Rhithrogena* sp. (mayflies) and *Tipula* sp. (craneflies) had levels higher than 9 $\mu g.g^{-1}$ dry weight and therefore may pose the highest risk to fish and birds. In the case of toxicity to insects, literature threshold levels are highly variable and therefore, it is difficult to make any assessment regarding the potential risk to insects. It should be noted that the threshold levels depend on many factors including the species of organism, developmental stage and the chemical form of selenium available.

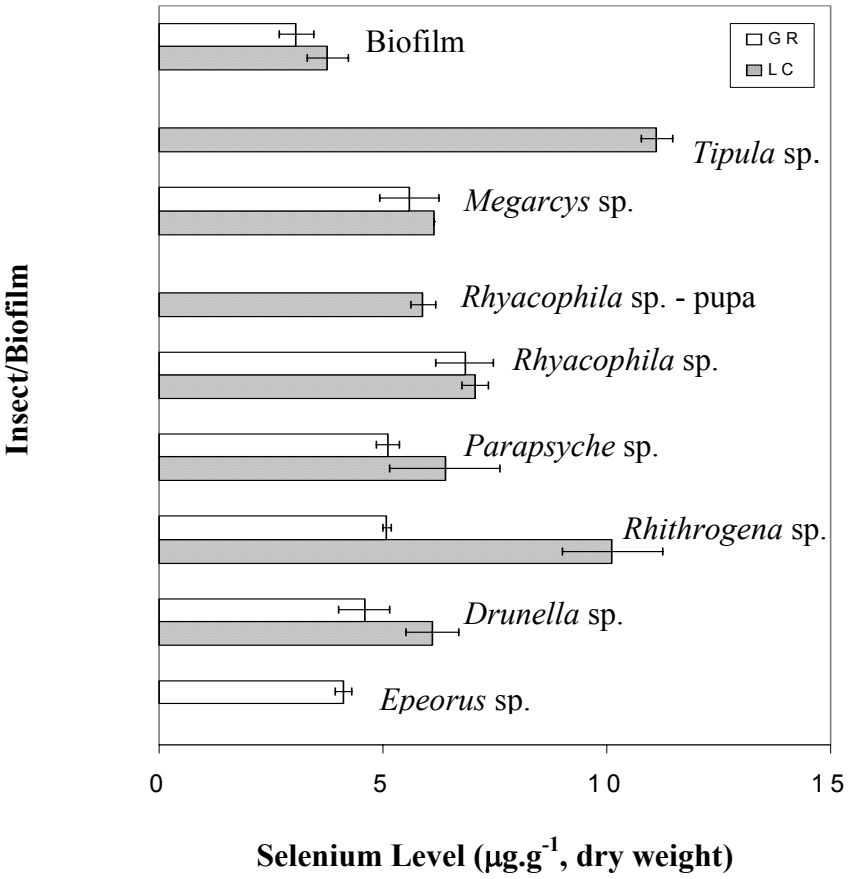

Figure 3.4 Total selenium levels (µg.g^{-1}, dry weight) in insects and biofilm samples collected from Luscar Creek (LC) and Gregg River (GR). *Epeorus* sp., *Drunella* sp., *Rhithrogena* sp. (mayflies); *Parapsyche* sp., *Rhyacophila* sp., *Rhyacophila* sp.- pupa- (caddisflies); *Megarcys* sp. (stoneflies); *Tipula* sp. (craneflies). Results are based on two independent measurements of each sample. Bars indicate ± standard error

3.3.2 Selenium Speciation

Selenium K near-edge X-ray absorption spectroscopy was used to determine the chemical form of selenium within the insects and biofilm samples. The selenium K-edge spectra are sensitive to the local electronic environment of the selenium (Figure 3.5) and thus can be used to identify the chemical type of selenium present. In this study, selenomethionine, trimethylselenonium and selenocystine are used to represent the selenium environments of R-Se-R, R_3Se^+, and R-Se-Se-R, respectively. The spectra of insects and biofilm samples show variability in the selenium species present in the samples tested (Figure 3.6). When spectra of samples from the stream sites were fitted to the sum of spectra of standard species (Figures 3.6 and 3.5, Table 3.2), organic selenides (R-Se-R), modeled as selenomethionine, were found to be the most abundant of all selenium compounds tested, ranging from 36% - 98%. Together with organic diselenides (R-Se-Se-R), these organic forms account for more than 85% of the selenium in all nymphal and larval insects measured. Selenate, the form of selenium most likely to be found in the aerobic aqueous environments, was not found to be a significant form of selenium in insects. However, small fractions of selenite were found in all organisms.

The biofilm samples are composed primarily of periphytic algae, but probably also contain bacteria, fungi, sediments and microinvertebrates. The biofilm samples show the highest percentage of selenite and also contain a moderate amount of elemental selenium, which was not found in any of the insect samples except in stonefly exuvia. Formation of elemental selenium in the environment may also occur through microbial reductions (1). Elemental selenium has low solubility and therefore low bioavailability, therefore, the presence of elemental selenium means that this fraction of selenium is relatively biounavailable, not only to the organisms that feed on it but also to higher trophic levels. A

measurement of the total selenium alone will overestimate the bioavailable selenium present in this case.

The distribution among chemical forms of selenium found in an insect species will depend upon many factors including the insect's feeding habit, the specific composition of the food, the proportion of the selenium that is absorbed, and whether the insect biotransforms the selenium. Primary consumers such as mayflies contain 9 to 11 % of selenium as selenite, possibly taken up in the course of normal feeding by scraping biofilm from rocks. The three different mayfly species recorded (Figure 3.6 a-c) showed very similar selenium speciation, perhaps a reflection of a constant selenium composition in their food. By contrast, filter-feeding caddisflies show significant variations between the selenium spectra and hence speciation between samples (Figure 3.6 d-f), perhaps due to differences in available food between sampling sites. *Parapsyche* sp. from Luscar Creek and Gregg River show 12% and 8% of selenium as selenite respectively. Predatory forms such as *Rhyacophila* sp. and stoneflies contain the lowest fraction of selenite. However, *Tipula* sp. in this study showed somewhat different selenium distribution compared to predators and most likely represents a detritivorous feeding habit, which is common among craneflies in Alberta streams. The variation in selenium fractions among insects may be a result of differences in the digestive biochemistry of insects adapted for different food sources (22) and or differences in selenium speciation in the food. The increase in fraction of organic selenium (and concomitant decrease in selenite) with the progression up the food web from primary consumers to predators suggests more complete metabolism of selenium species into organic selenium with increasing trophic level (Table 3.2). This effect constitutes a slight biomagnification of organic selenium species.

Exuvia (molted skins) of stoneflies and pupae of caddisflies were present in sufficient quantities at the Luscar Creek site to be sampled in this study. Molting and metamorphosis represent significant milestones in the life cycles of insects and

also represent opportunities for the insect to excrete selenium, since selenium in the molted skins is removed from the insect. In the case of the stonefly exuvia, we found a selenium composition consisting of organic selenides as the major component with minor components of selenite and elemental selenium. This shows 83% similarity to the composition of the stonefly larvae found at the same site, suggesting that there is only a small change in composition between the selenium retained in the tissues and the selenium excreted in the exuvia by molting. In contrast, the caddisfly pupa showed quite a different selenium speciation to that of the larva, with only 66% similarity. The pupa contained a significant proportion (30%) of a species, modeled as trimethylselenonium, which was not found in any of the other insect samples in this study. This observation agrees with an earlier study on selenium in a terrestrial insect, where trimethylselenonium-like species were observed in the pupa (19). Such methylated selenium species can act as a precursor in the synthesis of volatile species such as dimethyl selenide and its presence may indicate that volatile selenium has been produced and released. The conversion to a volatile species may indicate an active process to eliminate selenium or could be a secondary effect of metamorphosis. These observations strengthen the previous suggestion (19) that insects eliminate selenium by methylation and volatilization at the pupal stage, which would reduce selenium toxicity in adult insects. Additionally, such volatilization of selenium by insects contributes to volatilization from other sources including sediments, aquatic plants, and water (24)

Organic selenides are the most abundant fraction in all of the insect samples, as was previously seen for a terrestrial insect (19). The identification of organic selenides in stream insects warrants special attention since the common organic selenide, selenomethionine has been shown to cause toxicity in birds and fish in laboratory feeding studies (25). Bioaccumulation of selenomethionine occurs from diet to bird eggs (26). At dietary concentrations as low as 8 $\mu g.g^{-1}$ in the diet, selenomethionine caused embryonic deformities, teratogenic effects or

mortality of bird embryos (26, 27). Concentrations of organic selenides in insects from the current study approach this threshold value; for example, *Megarcys* sp. from LC was contained approximately 6 $\mu g.g^{-1}$ selenomethionine. In contrast, dietary intake of the diselenide, selenocystine at 16 $\mu g.g^{-1}$ or inorganic selenium as sodium selenite at levels up to 25 $\mu g.g^{-1}$ did not result in significant accumulation of selenium in bird eggs, nor were adverse reproductive effects observed (25, 26). In fish, both enhanced and reduced bioaccumulation of selenomethionine relative to naturally-incorporated selenium in fish meal has been reported (27). However, the toxic effects of other organic forms of selenium are less well documented and further work is needed.

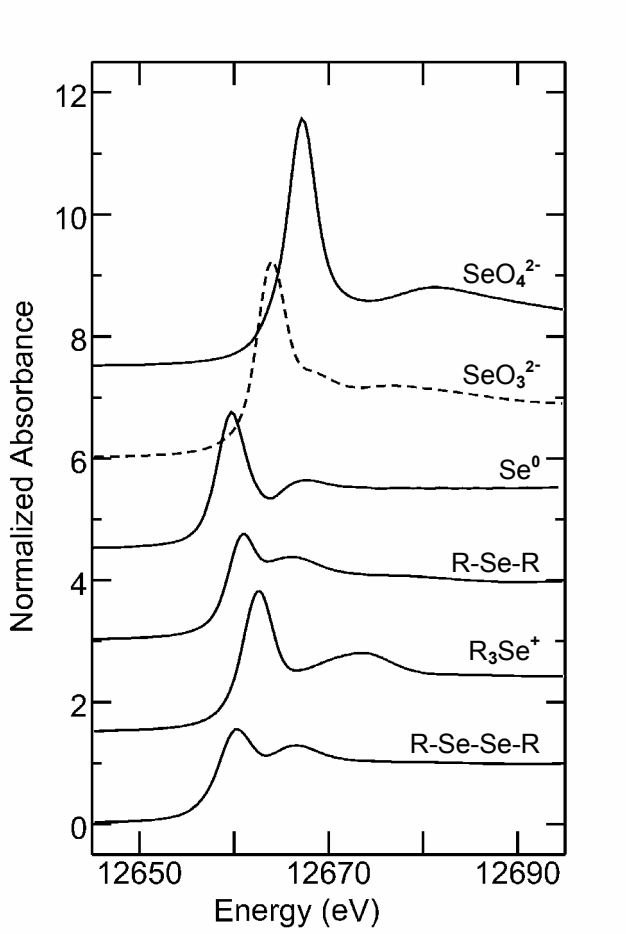

Figure 3.5 Selenium K near-edge spectra of standard selenium species. SeO_4^{2-} (selenate), SeO_3^{2-} (selenite), Se^0 (elemental selenium), R-Se-R (selenomethionine), R_3Se^+ (trimethylselenonium), R-Se-Se-R (selenocystine). SeO_3^{2-} is shown in broken lines to differentiate it from other overlapping spectra.

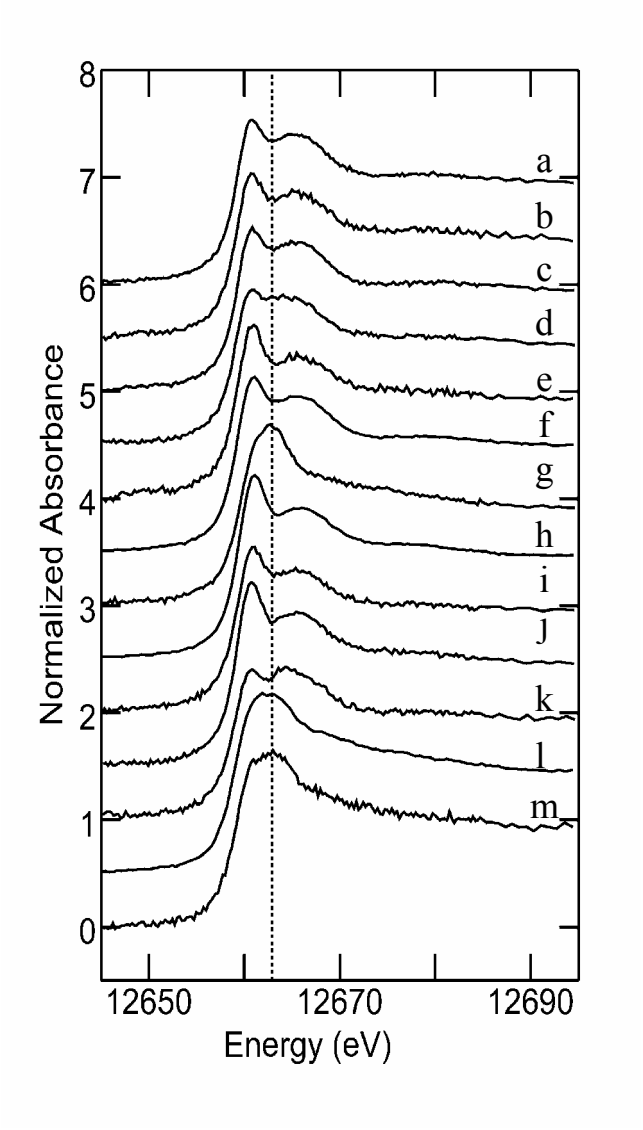

Figure 3.6 Selenium K near-edge spectra of insects and biofilm samples. Mayflies from Gregg river- *Epeorus* sp. (a), *Drunella* sp. (b), *Rhithrogena* sp. (c); caddisflies- *Parapsyche* sp. from Luscar Creek (d), *Parapsyche* sp. from Gregg River (e), *Rhyacophila* sp. from Luscar Creek (f), pupa of *Rhyacophila* sp. (g); stoneflies- *Megarcys* sp. from Luscar Creek (h), *Megarcys* sp. from Gregg River (i), exuvia of stoneflies (j); craneflies- *Tipula* sp. (k); biofilm samples from Luscar Creek (l), biofilm samples from Gregg River (m). Dotted vertical line helps to emphasize the variation in the near edge.

Table 3.2 Percent of selenium species in insects and biofilm

Sample	SeO_3^{2-}	R-Se-R	R-Se-Se-R	R_3-Se^+	Se^0
Epeorus sp. (GR)	9(1)	67(3)	24(3)	0	0
Drunella sp. (GR)	10(1)	69(1)	21(1)	0	0
Rhithrogena sp. (GR)	11(1)	68(3)	21(3)	0	0
Parapsyche sp. (LC)	12(1)	54(3)	34(3)	0	0
Parapsyche sp. (GR)	8(1)	80(1)	12(2)	0	0
Rhyacophila sp. (LC)	7(1)	75(6)	18(3)	0	0
Rhyacophila sp.-pupa (LC)	11(1)	49(3)	10(3)	30(1)	0
Megarcys sp. (LC)	2(1)	98(1)	0	0	0
Megarcys sp. (GR)	5(1)	73(3)	22(3)	0	0
Exuvia of stoneflies (LC)	7(1)	81(3)	0	0	12(2)
Tipula sp. (LC)	15(1)	36(3)	49(3)	0	0
Biofilm (LC)	14(1)	52(3)	0	17(1)	17(1)
Biofilm (GR)	16(3)	52(3)	0	16(3)	16(3)

Edge fitting results of insects and biofilm samples from Luscar Creek (LC) and Gregg River (GR). *Epeorus* sp., *Drunella* sp., *Rhithrogena* sp., (mayflies), *Parapsyche* sp., *Rhyacophila* sp. (caddisflies). Values derived from percentage contributions of spectra of standards to the best fit of the sample spectra. Figures in parenthesis show three times standard deviation. Selenite, selenides, diselenides, trimethylselenonium and elemental selenium are represented by SeO_3^{2-}, R-Se-R, R-Se-Se-R, R_3Se^+ and Se^0, respectively.

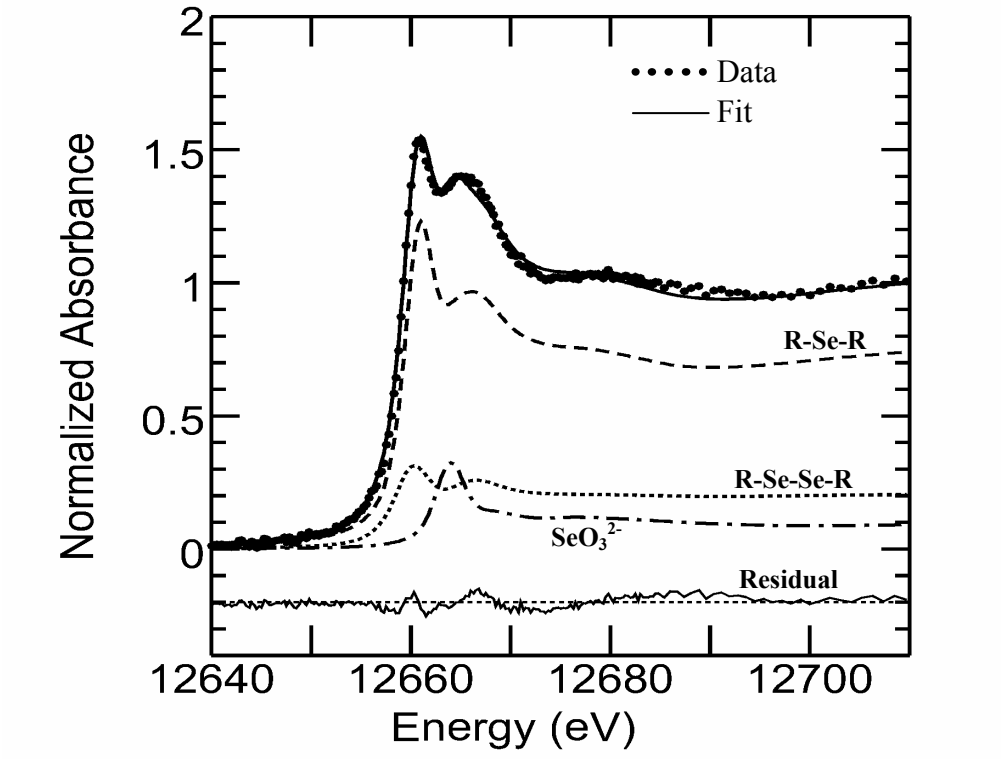

Figure 3.7 Least square fit of the spectrum of mayflies (*Epeorus* sp.) from Luscar Creek to the sum of spectra of standard selenium species. Figure shows the mayfly data, the fit and the individual components, scaled according to their contributions to the fit: SeO_3^{2-} (selenite), R-Se-R (selenides), R-Se-Se-R (diselenides). The residual is shown offset below. Numerical results are shown in Table 2.

3.4 Conclusions

Many insects in this study are prey for higher trophic level organisms such as fish or birds. Occurrence of selenite is evident in all organisms examined in this study. The formation of methylated forms of selenium and evidence for volatilization and immobilization of selenium are also observed. In deducing the chemical form of selenium in insects, we are showing the form of selenium in the diet of insectivores. We observe variations in selenium speciation among different insects, which may be attributed to the insects' feeding habits. These results suggest that a measurement of the abundance of insects exhibiting a particular feeding habit could be an important indicator of the potential risk posed by these insects to higher trophic level animals in a particular environment. This is useful, as a measurement of insect abundance is more readily carried out than a speciation study. Further work is needed to understand whether this idea can be more generally applied.

This study has demonstrated the use of X-ray absorption spectroscopy to address a problem of a trace level environmental contaminant in biota under field conditions. X-ray absorption spectroscopy provides unique tools for characterization of selenium species in the insects and biofilm samples, essentially without any pretreatment. Knowledge of the speciation is critical for understanding a contaminant element's release, mobility, toxicity and bioavailability in the environment. Currently, total selenium levels in different environmental components such as water, sediments, macroinvertebrates and fish are the most commonly used criteria in assessing the environmental risk. However, as demonstrated by our study, information on the chemical form of selenium in the various environmental components is needed to give a more refined indication of the risk.

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4. OBSERVATIONS ON SELENIUM TOLERANT INSECTS INHABITING A SELENIUM HYPERACCUMULATING PLANT (*Astragalus bisulcatus)*

4.1 Introduction

Hyperaccumulation of metals and metalloids in plants is a fascinating phenomenon in which some plant species accumulate exceptionally higher levels of an element than other plant species growing in the same area (1). There are various hypotheses for the adaptive role of elemental hyperaccumulation in plants including allelopathy (inhibitory chemical is released into the environment by one plant affects the development and growth of neighboring plants), drought resistance and defense against herbivory or pathogens. Most evidence support the third, hypothesis of defense against predators, parasites and pathogens which has generated considerable interest. This idea has been termed the elemental defense hypothesis of metal hyperaccumulation (1).

Selenium hyperaccumulating plants occur commonly on seleniferous soil originated from selenium-bearing cretaceous sediments. They are also known as selenium indicator plants (2). Selenium hyperaccumulating plants such as *Astragalus* spp. and *Stanleya* spp. are able to accumulate selenium at concentrations up to 10,000 $\mu g.g^{-1}$ dry weight in shoots (4). Ingestion of these plants by animals can cause chronic or acute selenium poisoning known as alkali disease and blind staggers. The high accumulators like *Astragalus bisulcatus* (two-grooved poison milk vetch) [Fabaceae] can accumulate selenium up to 0.65% of its shoot dry biomass (4). *Astragalus bisulcatus* accumulates Se-methylselenocysteine mainly in young leaves (6, 7). In more mature leaves, Se-methylselenocysteine concentration is lower and selenate predominates (6). Se-methylselenocysteine is also an intermediate in the formation of volatile dimethyldiselenide in *A. bisulcatus*, which is responsible for the characteristic odor of these plants (8). These plants are

poisonous, affecting cattle, sheep and horses; in fact, *A. bisulcatus* is one of several plants called "locoweed". As little as 2 pounds can cause poisoning in mature cows within a few hours after being eaten. However, many insect species have successfully adapted to utilize these plants.

Insects attacking A. *bisulcatus* in Saskatchewan were recorded in early literature (9) suggesting that these insects may have adapted tolerance mechanisms against selenium. The objective of this study was to monitor the insects inhabiting different parts of the *A. bisulcatus* plant and to characterize the selenium chemical form using X-ray absorption spectroscopy.

4.2 Materials and Methods

4.2.1 Insect Sampling

Astragalus bisulcatus plants growing on a river bank at University of Saskatchewan campus were surveyed for insects during the 2005 and 2006 growing season. Plants were located on a westerly facing edge of the riverbank and on the sloping sides of the bank. Three plants on the edge of the riverbank were surveyed for insects during the course of this study. Insects were sampled and rapidly frozen in liquid nitrogen for XAS analysis as described in Chapter 2.

4.2.2 X-ray Absorption Spectroscopy

Frozen insects, 5 insects per sample, were ground in liquid nitrogen, placed in 2mm sample cells and kept frozen in liquid nitrogen for XAS analysis. Parasitoid cocoons were packed directly in the cells and frozen in liquid nitrogen. XAS data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 9-3 as described in Chapter 2. Spectra were also collected of dilute aqueous solutions of standard selenium species, buffered at pH 7. These included the inorganic selenium forms selenate (Se^{VI}) and selenite (Se^{IV}), the organic forms

selenomethionine, trimethylselenonium and selenocystine as well as solid elemental selenium (recorded in transmittance). Background subtraction, normalization and data analysis were carried out as described in Chapter 2.

4.3 Results and Discussion

4.3.1 Insects Found on *Astragalus bisulcatus*

A number of insects were utilizing various parts of the plant throughout the growing season. Larvae of Lycaenid butterfly [Lepidoptera: Lycaenidae] (figure 4.1 a) were found on both mature and young leaves of *A. bisulcatus*. In general, Lycaenids are small, delicate slender bodied butterflies. They are rapid fliers. Larvae are flattened and slug like, and many secrete honeydew, which attract ants. Five larvae were collected on May 24, 2006 and were reared in the laboratory for pupation and adult emergence. However, all larvae were parasitized by a wasp (Hymenoptera, Braconidae) (Figure 4.1b) and adult wasps emerged on June 12, 2006.

a **b**

Figure 4.1 a) Leaf feeding caterpillar, b) Caterpillar after parasitization (wasp cocoons on the side)

Leaf folding larvae of Diptera were confined to young leaves of *A. bisulcatus*. Each larva folds a young leaf along the midrib (Figure 4.2 a,b), seals it and develops inside the leaf. They were observed in mid June 2005 and 2006 on flowering plants. Larvae are legless, tiny (about 3 mm) maggots, with a small and poorly developed head.

a **b**

Figure 4.2 Leaf folding larvae (Diptera) a) folded leaves with larvae inside, b) larva

Seeds were heavily attacked by beetle larvae (Figure 4.3). Larvae developing inside the field-collected seeds were dissected on September 2005. In general, adults of seed beetles are stout-bodied beetles belong to the subfamily

Bruchinae (Chrysomelidae) and lay eggs on flowers or on developing pods. Larvae bore into the seeds and feed inside the seed and pupate in it. Some of the seed beetles can become serious pests attacking stored seeds.

Figure 4.3 Coleoptera larvae developing inside the seeds

In the course of these observations, a number of other insects were found on the foliage of *A. bisulcatus.* Plant bugs and thrips (Thysanoptera) (sucking insects), ladybugs (Coleoptera) (predators) were also observed. However, sampling was limited to the insects that feed exclusively on *A. bisulcatus* plants.

Selenium in plants has been shown to deter feeding by insects (9-12). However, tolerance of some insects to significantly higher levels of selenium in *A. bisulcatus* is interesting. Freeman et al. (13) compared the levels of selenium in different parts of *A. bisulcatus* and reported that young leaves had higher selenium concentration than older leaves and flowers, fruits and seeds had more selenium

than young leaves. These indicate that developing leaves and reproductive parts concentrated the highest selenium overall. Insects observed in this study are thriving on developing leaves and seeds of *A. bisulcatus.* Leaf folding Diptera larvae were confined to developing leaves of the plant. Although caterpillars did not seem to have preference for a particular plant part, Coleoptera larvae were confined to seeds within the pods. Trelease reported that Bruchid beetles of the species *Acanthoscelides fraterculus* (Coleoptera: Bruchidae) and seed chalcids, *Brachophagus meziconus* (Hymenoptera: Chalcididae) were able to feed on seeds of *A. bisulcatus* containing 1475 μg g^{-1} dry weight of selenium and to complete their life cycle (4). These insects were feeding on seeds containing about 70 times the concentration of selenium which is known to be lethal within a few weeks to rats, cattle, pigs, sheep and horse (4). It is interesting to note that caterpillars found on leaves of *A. bisulcatus* were parasitized by wasp larvae without any ill effects from selenium, extending the tolerance to all three trophic levels.

Using chemically specific XAS imaging, Pickering et al. showed that the young leaves of the *A. bisulcatus* plants contain organic forms of selenium almost exclusively, whereas the older, lower selenium leaves are much higher in selenate (6). Diptera larvae observed in this study always confined to young leaves probably taking advantage of organic forms of selenium. However, organic selenium species, Se-methyl selenocysteine and selenomethionine are known to cause opposite toxic effects in animals. Selenomethionine is directly incorporated in place of methionine into proteins, causing toxicity, whereas Se-methyl selenocysteine is less toxic.

4.3.2 Speciation of Selenium in Insects

Selenium K near edge spectra are given in Figure 4.4. All insects in this study were found to contain only organic forms of selenium with more than 96% of selenium in the form of R-Se-R and less than 4% of selenium in the form of R-Se-Se-R (Table 4. 1). Inorganic forms of selenium such as selenite or selenate were not detected as significant components. Although *A. bisulcatus* is known to accumulate Se-methylselenocysteine (4), and this compound may become bioavailable to insects, selenomethionine was used as the commercially available model compound. Since selenium environments of Se-methyl selenocysteine and selenomethionine are similar with both carrying $R\text{-}CH_2\text{-}Se\text{-}CH_3$ formula, near-edge spectra of these two compounds are expected to be similar which make it difficult to distinguish Se-methyl selenocysteine and selenomethionine (6).

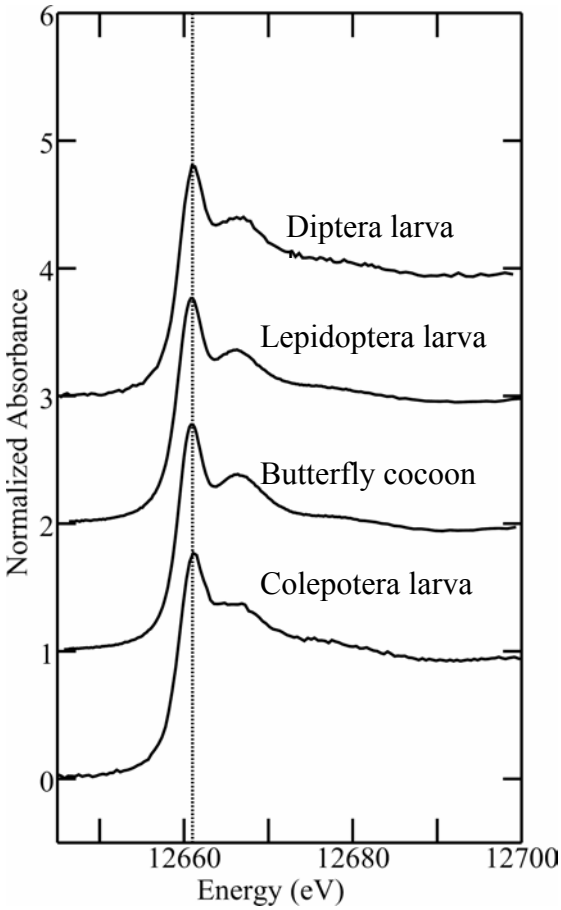

Figure 4.4 Se K near edge spectra of insects living on different parts of *Astragalus bisulcatus* plant

Table 4.1 Percent of selenium of insects and cocoons

Sample	R-Se-R	R-Se-Se-R
Diptera larvae	96(6)	4(1)
Lepidoptera larvae	100	0
Parasitoid cocoons	100	0
Coleoptera larvae	95(6)	5(1)

Edge fitting results of insect samples. Values derived from percentage contributions of spectra. Values derived from percentage contributions of spectra of standards to the best fit of the sample spectra. Figures in parenthesis show three times standard deviation. Selenides and diselenides are represented by R-Se-R and R-Se-Se-R, respectively.

4.4 Conclusions

Although many studies support the hypothesis that selenium hyperaccumulation functions as an elemental plant defense mechanism, some insects are able to overcome selenium toxicity and feed on various parts of *A. bisulcatus.* These insects carry organic forms of selenium with the majority of selenium is in the form of R-Se-R. However, it is not possible to draw a conclusion that the ability of insects to tolerate high levels of selenium is due to the uptake of less toxic selenium forms from the plants or special mechanisms developed by insects to detoxify selenium. Selenium in these insects may serve as a source of selenium to the local environment.

4.5 References

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5. TOXICITY AND BIOTRANSFORMATION OF ARSENATE IN INSECTS: EVIDENCE FOR A NOVEL ARSENIC COORDINATION IN BIOLOGY

5.1 Introduction

5.1.1 Sources of Arsenic in the Environment

Arsenic is the 20th most abundant element in the Earth's crust and 14th in seawater (1). Arsenic is released to the environment through natural weathering, volcanic activities, mining and mineral processing and fossil fuel combustion. Arsenic containing minerals such as arsenopyrite (FeAsS), realgar (AsS) and orpiment (As_2S_3) are associated with sulfide ores or other metal ores and play an important role in introducing arsenic to the environment (2). High levels of arsenic in tailings from copper, lead, gold and uranium mining and processing are of great environmental concern. In Yellowknife (Northwest Territories), both historical and recently deposited gold mine tailings contain arsenic levels as high as 89,000 μg/g (3). In northern Saskatchewan, arsenic levels in Rabbit Lake uranium mine tailings ranged from 56 to 6,000 μg/g (4).

Sources such as pesticides, herbicides and silvicides have increased the levels of arsenic in agricultural land (1, 5). Arsenic compounds such as Paris green (cuprous arsenite) was used to control colorado potato beetle (*Leptinotarsa decemlineata*) in the eastern United States (1). Lead arsenate has been a very effective insecticide for many years against codling moth (*Carpocapsa pomonella)* in apple orchards (1). In recent decades, the use of inorganic arsenicals has been replaced by organoarsenicals for herbicide application. Herbicides containing organoarsenicals in today's market include cacodylic acid (also known as dimethylarsinic acid) and its salts- monosodium and disodium methanearsonate (6).

Arsenic is used in glass manufacturing, laser materials, lead acid batteries and in producing color in fireworks (7).

Inorganic arsenic of geological origin is found in ground water which is used for drinking in several parts of the world including Bangladesh. In Bangladesh alone, 35 million people are exposed to arsenic contaminated drinking water (8) and human mortalities of 20,000 annually occur as a result of arsenic poisoning. Arsenic containing additives such as phenylarsonic acids are used in poultry feeds to increase growth rates, improve food utilization and enhance pigmentation. (6). In recent years, regulatory and public attention has focused on the potential risks associated with the use of arsenic, chromated copper arsenate in the preservation of timber (9). These arsenic containing wood preservatives are no longer used in Canada.

5.1.2 Effects of Arsenic in Organisms

Arsenic has no known beneficial role for humans or other animals. Toxic effects of arsenic have been demonstrated in humans and wildlife. Excessive arsenic exposure has been associated with increased incidence of skin, lung and possibly liver cancers in humans (10).

Arsenic has been observed to bioaccumulate particularly in lower level food-chain organisms such as shellfish (11), insects (5), and earthworms (12). Stoneflies accumulated up to 131 times the water concentration after 28 days of exposure (11). Elevated levels of arsenic have been found in other insects and in insectivorous birds in arsenic contaminated areas (5). Over two decades, arsenic based pesticides have been used in British Columbia to control mountain pine beetles and as a result, bioaccumulation of arsenic occurred in insects and insectivorous birds such as woodpeckers and chickadees (5). Insects are able to transport arsenic into different habitats. Immature stages living in contaminated terrestrial or aquatic habitats may carry arsenic long distances as they emerge as

flying adults. This movement is exemplified by the bioaccumulation of arsenic by herbivorous larvae of bogong moths (*Agrotis infusa*) [Lepidoptera: Noctuidae] in Australia (13). Spring generation of adult bogong moths migrate more than 1000 km to aestivate in mountain regions of eastern Australia. Migratory moths contain high levels of arsenic and their carcasses caused toxicity to vegetation (13). Therefore, understanding the effects of arsenic on insects is important with regard to assessing the ecological risks posed by arsenic in the food chain.

Once taken up by the larvae during feeding, arsenic may transfer into other life stages of insects such as pupae and adults living away from the point sources. It is possible that insects are able to eliminate some arsenic through excretion. Both of these options have interesting effects on the environment. However, the transfer of arsenic between immature stages has received very little attention. Studies involving sensitive life stages such as larvae, pupae and other stages would more accurately access the toxicity of arsenate to organisms. In this study, the effect of arsenate on the development of larvae, pupae and adult stages of bertha armyworm (*Mamestra configurata,* Walker) [Lepidoptera: Noctuidae] was examined under laboratory conditions.

5.1.3 Speciation of Arsenic in Insects and Excretory Products

Knowledge of biological processes that determine arsenic mobility and biotransformation in the environment is important for better understanding the impact of elevated arsenic levels in the environment. Further, the toxicity and bioavailability of arsenic in the environment depend critically on the chemical form in which it is presented. Under aerobic conditions, inorganic arsenic often is present predominantly in the pentavalent oxidation state as arsenate; however, reduced trivalent forms such as arsenite can occur and these can be significantly more toxic than arsenate. The dominant arsenic species found in apple orchards in

Massachusetts treated with arsenic based insecticides were found to be arsenate (14). Marine animals such as fish, crustaceans and mollusks contain predominantly trivalent arsonium species such as arsenobetaine and to a lesser extent arsenocholine whereas pentavalent arsenosugars are found naturally in marine algae (15). Arsenobetaine found in these animals is considered to be non-toxic (16, 17). Methylated trivalent arsenic species such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) are found in mammals and have recently been reported to be more toxic under some circumstances than inorganic arsenite (18), but more study is required to confirm this.

Synchrotron X-ray Absorption Spectroscopy was used to identify the chemical species of arsenic during larval, pupal and adult stages of bertha armyworm. In order to understand how larvae and pupae handle dietary arsenic, the chemical form of arsenic in fecal matter (frass) and molted skins of larvae (larval exuvia) and empty pupal cases (pupal exuvia) were examined as these provide possible routes of elimination of arsenic.

5.1.4 Micro X-ray fluorescence Imaging and X-ray Absorption Spectroscopy Imaging

The elements absorbed into an organism are distributed into different areas including the site where they cause the damage. In an effort to understand the organs responsible for arsenic absorption, biotransformation and toxicity, we also used XAS imaging with micro X-ray fluorescence imaging (μ-XRF). This allowed us to determine the microscopic distribution of arsenic chemical forms and other elements within intact larvae. X-ray Absorption Spectroscopy imaging has not been carried out on insects, and relatively little on organisms in general, although μ-XRF imaging of selenium localization was carried out on high selenium-fed diamondback moth larvae (*Plutella xylostella*) (19)

5.2 Materials and Methods

5.2.1 Biology of the Insect

The bertha armyworm (*Mamestra configurata* Walker) is native to North America and a pest of a wide variety of broadleaved plants including canola, alfalfa and flax (20). Bertha armyworm is used as a model insect in various physiological and biochemical studies. Its biology and ecology are well known and it can be successfully reared in the laboratory on a synthetic diet.

Bertha armyworm overwinters as pupae in the soil, and the adults emerge from early June until early August. Females lay eggs in masses of 20-200 on the underside the leaves of Brassicaceae plants. Larvae hatch in about 1 week after the eggs are laid and immediately begin feeding on the foliage. Bertha armyworm has 6 instar stages and the final two instars preferentially feed on the developing pods. Under field conditions, larvae take approximately 6 weeks to complete the development (20).

5.2.2 Insect Rearing

Bertha armyworm larvae were reared in plastic diet cups containing alfalfa based, Bucher and Bracken diet (21) and maintained at a constant temperature of 21 ± 1 °C, 60 % relative humidity, and a photoperiod of 20 hours of light followed by 4 hours of darkness (20:4 L:D). Sodium arsenate (Na_2HAsO_4) was dissolved in the prepared diet before pouring into the cups. Larvae were introduced to the diet at the neonate (newly hatched, 1st instar) stage, and transferred to new diet cup twice a week (Figure 5.1 a) until the 6^{th} instar larvae attain pupation. Late 5^{th} instar larvae are shown in Figure 5.1 b.

5.2.3 Preliminary Study on the Dosage of Arsenate

A preliminary study was conducted to determine a level of dietary arsenate that produced low but measurable toxicity and levels of arsenic that were measurable by X-ray absorption spectroscopy. First and third instar larvae were reared on a diet containing 1000 μM, 100μM or 10 μM of sodium arsenate or arsenate-free diet (100 larvae per treatment). After 7 days, the mortality of the arsenate-fed larvae was compared (Table 5.1). Surviving larvae from the arsenate treatments were used for X-ray absorption spectroscopy measurements as shown in the chapter 2. The level of arsenate to be used in the subsequent experiments was based on the results of Table 5.1.

a **b**

Figure 5.1 a) Diet cups showing insects, b) 5th instar bertha armyworm larva on a canola stem

Table 5.1 Survival and development of arsenate treated 1st and 3rd instar larvae from the preliminary study

Treatment	1000 µM		100 µM		10 µM		Control	
	1st instar	**3rd instar**	**1st instar**	**3rd instar**	**1st instar**	**3rd instar**	**1st instar**	**3rd instar**
% Survival	52	69	86	100	97	100	100	100
Instar Stage after 7 days	1st	3rd	3rd	4th	3rd and 4th	5th	3rd and 4th	5th

Observations made on 100 larvae from each instar stage 7 days after introduction to the diet.

Spectra from the larvae collected from all three treatments showed similar arsenic species (data not shown). Insects fed with 1000 µM of arsenate had the highest mortality (52 % survival) and therefore this treatment was not included in additional experiments. Larvae reared on 100 µM arsenate showed good survival (86 %) and gave a more adequate (stronger) signal in X-ray absorption spectroscopy than the larvae reared on 10 µM arsenate (97 % survival). Therefore, 100 µM of arsenate was used in subsequent studies. X-ray absorption spectroscopy of the surviving larvae from all three concentrations (data not shown) showed similar speciation to each other and to the study described below, indicating that the dietary concentration did not have a substantial effect on the biotransformation at these levels.

X-ray absorption spectroscopy of the diet containing 0.1 mM arsenate, frozen in liquid nitrogen 24 hours after preparation, indicated that arsenic in the diet remained as arsenate and the preparation and storage of the diet did not significantly change the chemical form.

5.2.4 Toxicity of Arsenate to Bertha Armyworm

Neonate larvae were fed with 100 μM arsenate, and arsenate-free control diet. There were five replicates, each consisting of five cups with four larvae in each cup, giving 20 larvae per replicate and 100 larvae per treatment. Larval survival to pupation, time taken to reach pupation, pupal weight and time to reach adult emergence were recorded. The results were analyzed by one way analysis of variance (ANOVA) (22).

5.2.5 Determination of Total Arsenic using Atomic Absorption Spectroscopy

5^{th} instar larvae, pupae and adults, 10 from each stage, were sampled for total arsenic analysis using hydride generation atomic absorption spectrometry. Insects were prepared for analysis as described in chapter 3. Two independent measurements were made of each sample and then averaged.

5.2.6 Arsenic Speciation in Insects using X-ray Absorption Spectroscopy (XAS)

Arsenic treated insects were collected for XAS studies as follows: larva (ten 5^{th} instar larvae, 2 larvae/replicate); pupa (ten pupae, 2 pupae/replicate); and adult (five males and five females of newly emerged moths, 1 moth/replicate). Samples were rapidly frozen and stored in liquid nitrogen. They were then finely ground under liquid nitrogen, packed in 2 mm pathlength sample cuvettes, and kept frozen in liquid nitrogen until data collection.

X-ray absorption spectroscopy data were collected on beamline 9-3 at the Stanford Synchrotron Radiation Laboratory (SSRL) as described in chapter 2. Data analysis was carried out according to the standard methods using the EXAFSPAK program suite described in Chapter 2. Spectra of standards were collected in fluorescence of dilute aqueous solutions (1-5 mM) of arsenic species including the inorganic arsenic forms, arsenate at pH 9 and arsenite at pH 7, as well as the aqueous thiolate-coordinated arsenic As^{III}-*tris*-glutathione. In addition, spectra of methylated arsenic compounds including monomethylarsonic acid (MMA^{V}), dimethylarsinic acid (DMA^{V}) and arsenobetaine were initially included in the fits and later rejected as they were not significant components in the sample spectra.

5.2.7 Molecular Form of Arsenic in Excretory Products of Larvae

In this study, X-ray Absorption Spectroscopy was used to understand the molecular form of arsenic in the excretory products of the bertha armyworm larvae. Molted skins (exuvia) of forty 4^{th} and 5^{th} instar larvae and feces (frass) of 10 larvae were collected within 24 hrs. Samples were packed in 2 mm sample cells, frozen in liquid nitrogen and speciation was determined using XAS as described in chapter 2.

5.2.8 Preparation of Standards

Solutions of arsenic standards were prepared from sodium arsenate (Na_2HAsO_4), glycerol ($C_3H_8O_3$), catechol ($C_6H_6O_2$) and glutathione ($C_{10}H_{17}N_3O_6S$) obtained from Sigma-Aldrich, Canada. The arsenic-catechol solution was prepared by adding excess catechol to sodium arsenate with subsequent acidification to pH 2. The arsenic-glycerol complex was prepared as a solution of arsenate, acidified to pH 0, in the presence of excess glycerol. As^{III}-*tris* glutathione was prepared by adding excess glutathione to sodium arsenite and the solution was maintained at pH 7. With the exception of the arsenic-catechol complex, 30% (v/v) glycerol was added to solutions as a glassing agent. All standards were pipetted into 2-mm path-

length Lucite sample holders and frozen in liquid nitrogen with the exception of the arsenic-catechol complex which was flash-frozen in a slurry of isopentane and liquid nitrogen before transferring to liquid nitrogen.

5.2.9 Micro X-ray fluorescence Imaging and X-ray Absorption Spectroscopy Imaging

Micro X-ray fluorescence imaging (μ-XRF) and chemically specific imaging were conducted on arsenate (100 μM) fed 3rd instar bertha armyworm larva using the methods previously described in Chapter 2. The distributions of other metals such as copper and zinc were also monitored simultaneously with those of arsenic, to observe possible co-association of these metals with arsenic. Following the collection of maps of arsenic species, arsenic K-edge micro X-ray absorption spectroscopy (μ-XAS) was performed at selected pixels to corroborate the arsenic chemical speciation.

5.3 Results and Discussion

5.3.1 Toxicity of Bertha armyworm to Arsenate

Observations on the development of the arsenate-fed larvae, through their metamorphosis to pupae, and their eventual emergence as adults were made in comparison with larvae fed on an arsenic-free diet. The days to pupation, larval survival, pupal weight and days to adult emergence for bertha armyworm are shown in Table 5.2. Development times of arsenate-treated and untreated larvae were not significantly different. When compared with untreated larvae, survival of arsenate-fed bertha armyworm larvae was significantly reduced, although a survival of 79% was still achieved (Table 5.2). For the surviving larvae, pupal weight was not significantly affected by the arsenate treatment, whereas duration of the pupal stage was significantly longer for the arsenate-treated larvae.

Data on the effects of arsenic on fish and invertebrates indicate that many species are capable of tolerating elevated levels of arsenic (23). Robertson and McLean observed that the larvae of the western spruce budworm (*Choristoneura occidentalis*) can feed on As^{III} contaminated vegetation up to 3,300 $mg.g^{-1}$ dry weight (24). Stoneflies, trout and snails did not show adverse effects to inorganic and organic arsenic at 100 and 1000 $\mu g.l^{-1}$ level. (11). However, toxicity and mobility of arsenic largely depend on the chemical speciation.

Table 5.2 Effect of arsenate on bertha armyworm survival and development

Treatment	Larval Survival (%)	Days to pupation Larvae-Pupae	Pupal Weight (g)	Days to adult emergence (Pupae- Adults)
Arsenate	79.0 ± 1.0^{b}	27.4 ± 0.2^{b}	0.35 ± 0.01^{a}	23.4 ± 0.2^{a}
Control	92.0 ± 1.2^{a}	27.0 ± 0.0^{b}	0.35 ± 0.01^{a}	21.2 ± 0.2^{b}

Table shows mean ± standard deviation for n=5 replicates (20 larvae per replicate). Means followed by the same letter (^{a,b}) within a column are not significantly different (Tukey's test, $p \leq 0.05$).

5.3.2 Total Arsenic Analysis in Insects

Atomic absorption spectroscopy results showed that the larvae fed with arsenate accumulated 18 ± 1 $\mu g.g^{-1}$ (dry weight) of arsenic and during subsequent development, pupae and adult contained arsenic levels 11 ± 2 and 7 ± 2 $\mu g.g^{-1}$ (dry weight) respectively (Table 5.3). In an earlier study (24), larvae of chironomids (Diptera, Chironomidae) exposed to zinc, cadmium and copper ions showed a decrease in these elements during metamorphosis. Loss of metals or metalloids by

shedding of the exoskeleton is the most likely explanation for reduction in levels during metamorphosis of insects (24, 25). However, the presence of arsenic in the flying adults, albeit at a lower dry weight level than in the larvae, may pose a risk for insectivorous predators living away from the point sources and deserves more attention.

5.3.3 Arsenic Speciation in Insects and Excretory Matter

Figure 5.2a-c shows the X-ray absorption near-edge spectra of selected aqueous arsenic standards. Arsenic K-edge spectra are dominated by dipole-allowed transitions of the core 1s electron to unoccupied bound states with a lot of 4p orbital character, and the spectra thus are very sensitive not only to valence but to geometry and nature of the ligand (26). Figure 5.2 e-g shows the arsenic near-edge spectra of standard arsenic compounds and insect samples of larval, pupal and adult *M. configurata* in comparison with the arsenic in the diet (Fig 5.2.d). The spectrum of the diet confirms that arsenate is not transformed before consumption, and the spectrum of the larvae is quite different from that of the diet, indicating that arsenate supplied in the diet is biotransformed by the larva. In contrast, the spectra of pupa and adult are very similar to that of larva, indicating that the majority of arsenic retained within the insect body is not further modified during subsequent life stages. Indeed, principal component analysis showed that the three spectra from whole larvae, pupae and adults require one predominant component, with possibly a small contribution from a second component. The major component shows good correspondence with the spectrum of As^{III}-*tris*-glutathione ($As^{III}(GS)_3$), in which reduced arsenic (As^{III}) is bound to three thiolate sulfur atoms from the cysteine peptide in glutathione (27). However, X-ray absorption spectroscopy is not generally sensitive to very long range structure and $As^{III}(GS)_3$ is used as a model for trivalent arsenic coordinated to three aliphatic thiols, $As^{III}(SR)_3$, in general. Such species have been observed spectroscopically in *Brassica juncea* treated with

arsenate (26). When spectra of the insect samples were fitted to the sum of spectra of standard species (Table 5.3), larvae, pupae and adult samples showed a small percentage of arsenite (ranging from 7-11 %) in addition to the major component of $As^{III}(SR)_3$.

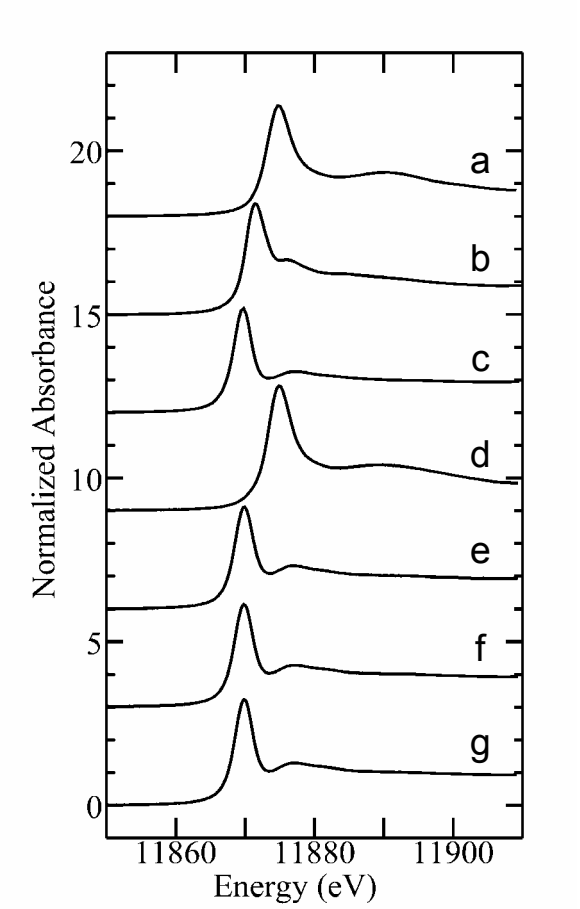

Figure 5.2 Arsenic K near-edge spectra of standards and spectra collected from insects. a) Arsenate at pH 9, b) arsenite at pH 7, and c) $As^{III}(GS)_3$, all as dilute aqueous solutions, in comparison with d) 100 µM arsenate in the diet, and e) larval, f) pupal and g) adult bertha armyworm reared on 100 µM arsenate. All spectra have been normalized to the edge jump.

Table 5.3 Concentration and chemical form of arsenic in bertha armyworm

Sample	Total Arsenic Level[A] [$\mu g.g^{-1}$ (dry weight)]	Percentage of arsenic[B] as:	
		Arsenite	$As^{III}(SR)_3$
Larva	18 ± 1	11 (2)	89 (2)
Pupa	11 ± 2	7 (2)	93 (1)
Adult	7 ± 2	8 (2)	92 (2)

[A] Total arsenic measured using atomic absorption spectroscopy (mean ± standard deviation, n=2).
[B] Percentage composition of arsenic chemical species, derived from the contributions of standard spectra to the fit of As K near-edge X-ray absorption spectra of insect samples. Figures in parenthesis show three times the estimated standard deviation of each parameter in the fit, derived from the covariance matrix.

The observed arsenic speciation has implications for the mobility, toxicity and bioavailability of arsenic within insects. As^{V} from the diet was reduced to As^{III}. Inorganic As^{III} as arsenite and some methylated species are generally accepted as the most harmful metabolites responsible for toxic and carcinogenic effects. However, methylated arsenic forms were not found to be significant in insects, and the predominant form was $As^{III}(SR)_3$ modeled as $As^{III}(GS)_3$. It has been shown that, in the presence of excess glutathione, arsenate is reduced by oxidation of glutathione and the resulting As^{III} is bound by additional glutathione molecules to form $As^{III}(GS)_3$ (27). Glutathione is a prevalent biological thiol, which plays a role in protecting the kidney from arsenite-induced toxicity (28). A similar protection mechanism may occur in insects resulting in lower toxicity in arsenate-fed insects.

The present study showed that $As^{III}(GS)_3$ is the main chemical form of arsenic in larvae, pupae and emerging adult insects. These insects may transfer $As^{III}(GS)_3$ to their predators and toxicity to predators may depend on their ability to tolerate $As^{III}(GS)_3$.

5.3.3.1 Arsenic K Near-Edge Spectra of Exuvia and Frass

Figure 5.3 A shows the As K near-edge spectra of arsenate-fed bertha armyworm larval frass and exuvia in comparison with intact larvae. The larval exuvia and frass show marked spectral differences compared with the whole larvae. In particular, both exuvia and frass show two major peaks at 11869.8 and 11876.3 eV. The lower energy peak corresponds well to the peak of $As^{III}(SR)_3$ which is the predominant form found in whole larva (Figure 5.3 Ab). The second peak occurs at a higher energy than that of the arsenate administered in the food and corresponds to a somewhat unusual form of oxidized arsenic(V). Speciation results are shown in Table 5.4.

Arsenic(V) coordinated by oxygen atoms usually exists as four-coordinate (tetrahedral) arsenate (Figure 5.3 Ba and Be). Near edge spectra of exuvia and frass show a prominent peak at 11876.3 eV, about 1.4 eV above the peak of four-coordinate arsenate (arsenate at pH 9). These spectra are in close agreement with solution spectra of arsenate equilibrated with glycerol (propane-1,2,3-triol) at pH 0 and with catechol (benzene-1,2-diol) at pH 2, representing aliphatic and aromatic vicinal hydroxyls, respectively (Figure 5.3 Bf and Bg). Detailed inspections suggest that the species in frass and exuvia is more similar to the glycerol, or other aliphatic complex. (29)

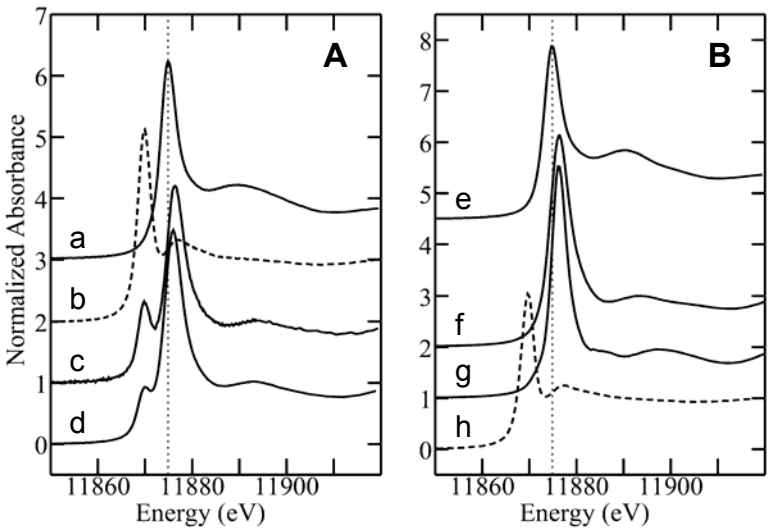

Figure 5.3 Arsenic K X-ray absorption near-edge spectra. (A) *M. configurata* a) diet, b) whole larva, c) larval exuvia and d) frass. (B) Aqueous solutions of e) arsenate, pH 9.0, f) arsenate-glycerol (pH 0), g) arsenate-catechol (pH 2), and h) As^{III}-*tris*-glutathione.

Table 5.4 Percentage of arsenic in exuvia and frass present as standard species determined by near-edge X-ray absorption spectroscopy

Sample	$[As^{V}O_6]$ [a] Arsenate (6-coordinate)	Arsenite [b]	As^{III}-*tris*-glutathione [c]
Larval exuvia	66(1)	0	34(1)
Frass	74(2)	7(3)	19(2)

Values derived from percentage contributions of spectra. Number(s) in parenthesis following a value represents three times the estimated standard deviation in the last digit(s) determined from the fit.

[a]Aqueous arsenate equilibrated with excess glycerol at pH 0.
[b]Aqueous arsenite, pH 7
[c] As^{III}-*tris*-glutathione

5.3.3.2 Extended X-ray Absorption Fine Structure (EXAFS)

Extended X-ray Absorption Fine Structure (EXAFS) was used to structurally characterize arsenic in larval frass and solution species (Figure 5.4); the exuvia signal was too weak to collect usable EXAFS. The best fits of the EXAFS spectra are shown as dashed lines in each case and the numerical results of the fit are shown in Table 5.5. The first coordination shell gives the most prominent Fourier transform peak in all cases (Figure 5.4 B). By contrast, As^{V}-glycerol and As^{V}-catechol complexes show 6 As–O at distances of 1.82 and 1.83 Å, respectively (29). This is in close agreement with known $As^{V}O_6$ species (30) with mean As–O distances ranging from 1.833 to 1.846. The frass also includes As–S at 2.25 Å, which is in excellent correspondence with the As–S distance of 2.24 Å determined in $As^{III}(SR)_3$ (Table 5.5). The first coordination shell approximately correspond to

55% of total arsenic present in $As^{V}O_6$ coordination, 20% as $As^{V}O_4$ and 25% as $As^{III}(SR)_3$ (Table 5.5).

The spectra of the glycerol and catechol complexes as well as the frass show additional weaker outer shell interactions. Distant As···C interatomic length is observed to be 2.74 and 2.69 Å, respectively, for As^{V}-glycerol and As^{V}-catechol complexes. The frass As···C distance of 2.74 Å indicates a closer similarity with the glycerol rather than the catechol complex, in agreement with the near-edge observations.

Currently the source of the vicinal di-hydroxyl chelator is unknown. However, the cuticle and midgut of insects, exoskeleton of many other invertebrates and cell wall of fungi contain polysaccharides such as chitin (31), which has an affinity to bind metals (32) and may involve in the formation of aliphatic 6-coordinated species. Involvement of aromatic groups may arise if arsenate binds with diphenolic compounds which often occur in abundance in insect cuticle (33).

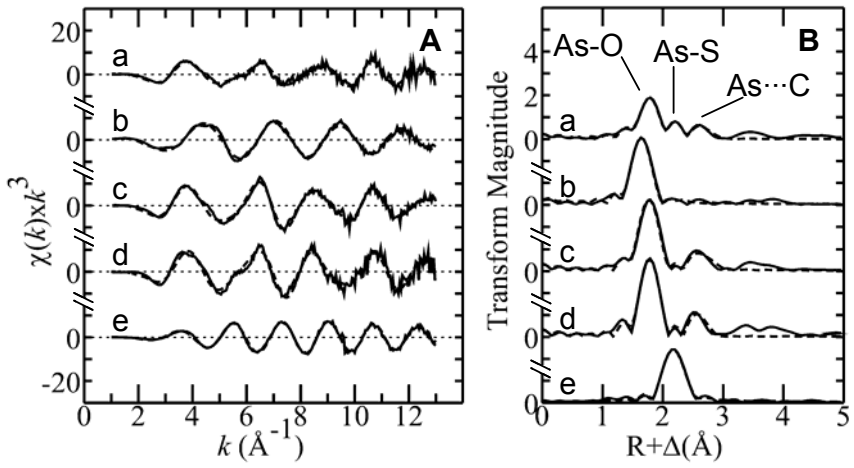

Figure 5.4 A, B- As K-edge EXAFS for arsenic in *M. configurata* frass and relevant solution species. a) frass, b) arsenate pH 9, c) arsenate-glycerol pH 0, d) arsenate-catechol pH 2, e) $As^{III}(SR)_3$ showing data (—) and best fit (– – –) as given in Table 2. EXAFS are k^3-weighted.

Table 5.5 Arsenic K-edge EXAFS curve-fitting results for arsenic in *M. configurata* frass and related solution species.[a]

Sample	As–X parameters				As···C parameters		
	X	N	R	σ^2	N	R	σ^2
Arsenate, pH 9.0	O	4.7(3)	1.688(2)	0.0036(5)			
$As^{III}(SR)_3$	S	2.9(2)	2.244(2)	0.0028(4)			
Arsenate-glycerol, pH 0	O	5.7(5)	1.819(3)	0.0037(6)	5(2)	2.74(1)	0.004(2)
Arsenate-catechol, pH 2	O	5.6(7)	1.826(5)	0.0029(9)	5(2)	2.69(2)	0.003(3)
Frass	O O S	3.2(5) 0.8(4) 0.7(2)	1.828(7) 1.688 [a] 2.247(18)	0.0027(12) 0.0036 [a] 0.0028 [b]	3(2)	2.74(2)	0.002(3)

[a] Type of bonded atom, X, mean coordination number, N, interatomic distance, R (Å), and Debye-Waller factor, σ^2 (Å^2). [b,c] Values fixed to those determined for [b] arsenate at pH 9 and [c] $As^{III}(SR)_3$. Values in parenthesis represent three times the estimated standard deviation determined from the fit.
Frass coordination numbers equate to approximately 55% [AsO_6], 20% [AsO_4] and 25% [AsS_3].

5.3.4 Localization of Arsenic Species and Other Elements using X-ray Absorption Spectroscopy Imaging

In XAS-imaging, the fine structure of the absorption edge is exploited to derive maps of the particular chemical forms of the element. Energies used for arsenic imaging were corresponding to the X-ray absorption of standard solutions of arsenate at 11874.9 eV (chemical form in the diet) and AsIII-*tris*-glutathione at 11869.7 eV (main chemical form in insects). Two fluorescence maps of arsenate

and As^{III}-*tris*-glutathione were collected on whole third instar larvae fed with 100 μM of arsenate. By using the intensities from the arsenic near-edge spectra of standards, the maps can be deconvolved to obtain quantitative concentration maps of arsenic species. This method has been described in detail in Chapter 2.

Figure 5.5 shows images of two different bertha armyworm larvae. First, an intact larva was imaged at 20 μm resolution to give an overview of the entire organism (Figure 5.5 a-c). A second larva was then imaged at 10 μm resolution (Figure 5.5 d-f) to provide more details on the region of the midgut, as described below. The larvae are both in lateral orientation and Fig. 5.5a and 5.5d show the X-ray transmittance of both larvae, which gives an indication of the amount of material in the projection.

Figure 5.5b and 5.5e show the localization of $As^{III}(SR)_3$, which is the predominant form of arsenic in the larvae. Arsenic as $As^{III}(SR)_3$ was detectable in the midgut area of the larvae with distinctly high localization towards the middle area of the midgut. (Figure 5.5 b,e). Relatively low levels of arsenic as $As^{III}(SR)_3$ were measured in the foregut and anterior part of the midgut region. The presence of As-S complexes in the intestine has been well documented in earthworms (12). Similarly, previous studies have shown that some metals tend to be concentrated in the insect midgut (33) Depuration of metals from the gut may occur when these storage structures are excreted from the body (34). The presence of metals and metalloids in the gut may also be favored by their binding to the insect peritrophic membrane which is a protective chitinous sheet lining the midgut. In Lepidoptera, the peritrophic membrane is produced continuously by the midgut and excreted with frass (34, 35).

In contrast to $As^{III}(SR)_3$, arsenic as arsenate was found only diffusely distributed throughout the larval body (Figure 5.5 c,f), possibly on the exoskeleton. Similarly, arsenic appeared to be in the exoskeleton of aquatic nymphs belonging to the genus *Hexagenia* (41). Arsenate is additionally found, albeit at low levels, in the

anterior foregut (Figure 5.6 c), a region which is relatively low in $As^{III}(SR)_3$. The same area is shown in Fig. 3, in which a single μ-XAS dataset from this region shows a small but significant fraction of arsenate (Figure 5.6a,c), and may correspond to undigested arsenate in the diet. This arsenate region is located directly anterior to the region which shows highest $As^{III}(SR)_3$ and essentially no arsenate (Fig. 5g,h, Fig. 6b,c). This may suggest that the high $As^{III}(SR)_3$ region is the site of reduction and complexation.

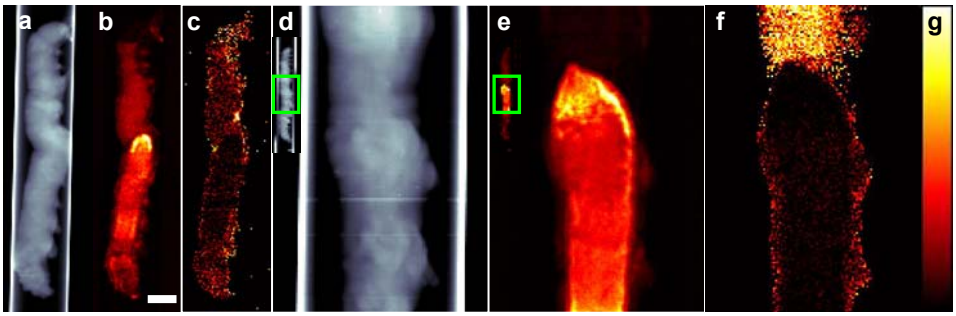

Figure 5.5 X-ray absorption spectroscopy imaging of arsenic in bertha armyworm larvae. a-c) Scan of complete larva using 20 µm pixel size; scalebar represents 400 µm. d-f) Detailed scan of second larva using 10 µm pixel size; scalebar represents 200 µm; insets d,e) location of scan on larva. Orientations are lateral, head up and mouth facing right. Intensity scale for b,c,e,f shown in g from minimum to maximum (white). a) and d) Sample thickness, relative scale from minimum to maximum (white). Bright vertical lines show the quartz capillary in which the larva was mounted. b) and e) arsenic as $As^{III}(SR)_3$, maxima b) 0.080 and e) 0.180 µm.cm^{-2}; c) and f) fraction of total arsenic as arsenate from 0 to 0.60

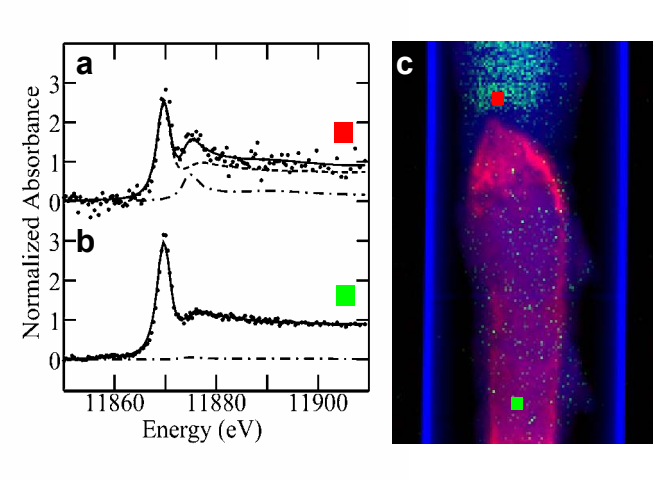

Figure 5.6 Micro X-ray absorption spectroscopy of arsenic in bertha armyworm. a,b) Arsenic K-edge X-ray absorption spectra showing data (•••) and best fit (——) to the sum of spectra of $As^{III}(SR)_3$ (– – –) and arsenate (– · – · –). Spectra were recorded from a single 10µm pixel in a) the posterior foregut, which fit to (21 ± 6) % arsenate; and b) the midgut with < 2 % arsenate, both with balance $As^{III}(SR)_3$. c) Map of $As^{III}(SR)_3$ (red) and arsenate (green) superimposed on X-ray transmittance (blue) using arbitrary intensity scales. The red and green squares identify the locations from which the spectra were recorded.

Figure 5.7 shows the distribution of manganese, iron, copper and zinc in bertha armyworm, each superimposed on the map of total arsenic. In insects, a variety of metals including zinc, manganese, iron and copper are found concentrated within the mandibles, ovipositors and various body parts (37-39). Insects also need small amounts of metals and metalloids as enzyme co-factors and as constituents for metalloenzymes. Both zinc and copper are found to be associated with the gut. Zinc is concentrated in regions of the midgut (Figure 5.7d, h). The observed profile of two parallel lines is expected for the projection image of a cylindrical structure and is consistent with the zinc being localized to the gut wall or lining. The arsenic

shows a similar profile in this region of the gut except that arsenic is concentrated more anterior, zinc more posterior. Copper is also localized in the gut area of the larvae with the highest level in the midgut region. In contrast to zinc, copper is present at lower levels and is not associated with the gut wall but instead is diffusely distributed. Slightly anterior to the midgut, another copper localized area is present on the ventral side of the larval body (Fig. 5.7c). Copper accumulation in the intestinal cells has been observed in larval *Drosophila* (29).

Zinc (Fig. 5.7d) is observed highly concentrated in the mandibles (which appear as two bright spots near the top of the image) in addition to the midgut. The presence of zinc in mandibles of herbivorous insects has been known for some time (37, 38) and reduces the abrasive wear that mandibles would suffer through chewing hard plant material. Enhanced levels of zinc have also been observed in grasshoppers and leaf-cutter ants where high levels of zinc are linked with greater hardness of the cuticle (37, 38). Manganese is also commonly found in insect mandibles. However, in the case of bertha armyworm, we find that manganese is not concentrated in the mandibles of the larvae, but is observed on the cuticle (Fig. 5.7a, e), possibly in association with pigment spots which are characteristic for this larvae. Potassium and calcium were seen in oral secretions (data not shown) but the low energy fluorescence of these elements combined with the fact that the larva was in a quartz capillary precluded additional detailed observations.

Iron was diffusely distributed, but additionally appeared in small areas of high concentration throughout the body (Fig. 5.7b, f). In general, insects acquire only a trace amount of iron, except for species that employ hemoglobin in respiration. Iron is involved in formation of insect cuticle (39). Iron containing magnetite (Fe_3O_4) has been found in the head and thorax of adult monarch butterflies, suggesting that the butterflies might use Earth's magnetic fields to navigate their migratory flights (40).

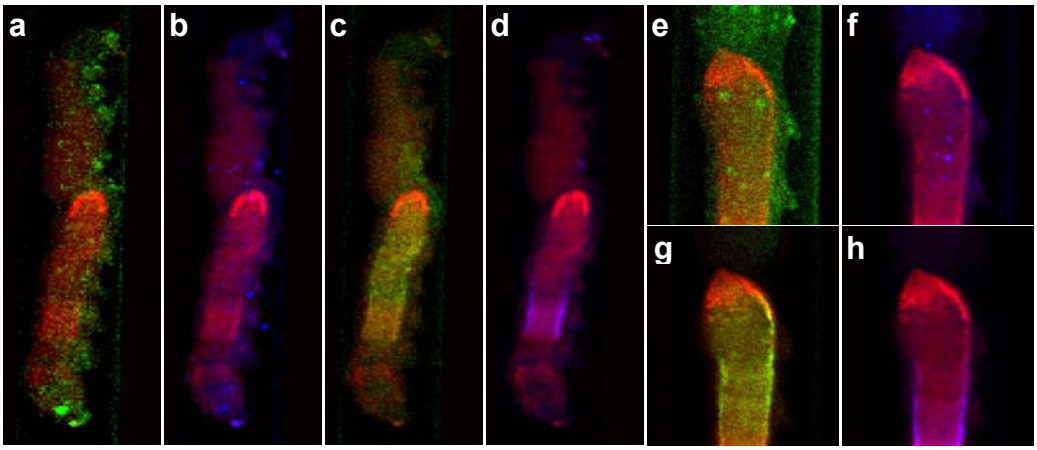

Fig. 5.7 X-ray fluorescence imaging of metals in bertha armyworm larvae. a,e) manganese (green); b,f) iron (blue); c,g) copper (green); d,h) zinc (blue), each shown in comparison with total arsenic (red). Intensity scales are arbitrary. Orientation, scale bars and resolutions as for Fig. 5.5

5.4 Conclusions

In summary, the present study provides new insights into the toxicity and biotransformation of arsenate in different life stages of an insect. Arsenate at 1000 µM level was lethal to early stages of the bertha armyworm larvae. Insects were much more tolerant and survived well on 100 µM and 10µM of arsenate.

X-ray Absorption Spectroscopy data showed that inorganic arsenic is biotransformed into organic species by insects. XAS analysis of the whole larvae,

pupae and adults indicate the presence of sulfur coordination following arsenic biotransformation in bertha armyworm larvae. XAS at the arsenic K edge showed that during metamorphosis, arsenic speciation varied only slightly from larvae to pupae and from pupae to adult stages of the insect. Bertha armyworm does not appear to form methylated arsenic species. This may be associated with the increased tolerance of insects to arsenic observed in this study.

Molting is a possible pathway for arsenic excretion in bertha armyworm larvae. It is evident that larvae are able to remove arsenic in fecal matter as well. A novel 6-coordinated form of arsenic is involved in both of these excretion processes. X-ray Absorption Spectroscopy imaging directly visualized the distribution of arsenic as arsenate and sulfur-coordinated arsenic forms in the larvae. Biotransformation of arsenate to $As^{III}(SR)_3$ occurred near the anterior part of the midgut. Presence of arsenate on the periphery of the larvae suggests possible storage mechanism on the exoskeleton. Zinc is seen closely associated with arsenic in the periphery of the midgut. Since arsenate does not exert very high toxicity on survival and development of the insects at this level, predators living in contaminated areas may not face scarcity of insects. However, the effect of arsenic-glutathione complexes on predators needs further understanding.

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6. EFFECT OF SELENATE AND A COMBINATION OF SELENATE AND ARSENATE ON INSECTS

6.1 Introduction

Although increase in selenium contamination in aquatic and terrestrial environments has been well documented and adverse effects on top predators such as fish and birds are known, information on toxic effects and selenium speciation in lower level food chain organisms is scarce. Previous studies have shown that immature stages of insects can accumulate substantial amounts of selenium (1, 2). Once taken up by the larvae during feeding, selenium may transfer into other life stages of insects such as pupae and adults living away from the point sources. It is possible that insects are able to eliminate some selenium through excretion. Both of these options have interesting effects on the environment. However, the transfer of selenium between immature stages has received very little attention, except for larvae and nymphs of some aquatic species (3). The importance of examining the toxicity of substances to a variety of life stages has been stressed (4) and information on the effects on different life stages can be used to identify vulnerable and tolerant links in the food chain.

Very little progress has been made to evaluate the impacts of metals or metalloids when they occur in the environments in mixtures. Although antagonistic effects of certain chemical forms of selenium and arsenic are known in mammals (5), the effects of combined action of selenium and arsenic on other animals are not known. Therefore, the aim of this study is to assess the possible effects of selenium alone and a combination of selenium and arsenic on larva, pupa and adult of bertha armyworm. X-ray Absorption Spectroscopy was used to study the selenium speciation in larvae, pupae, and adult stages and to study various excretory

pathways of larvae and pupae. The fate of selenate within the body of the larva was examined by using X-ray Absorption spectroscopy imaging.

6.2 Materials and Methods

6.2.1 Toxicity of Selenate and a Combined Treatment of Selenate and Arsenate to Bertha Armyworm

Observations on the survival and development of 100μM selenate-fed larvae, through their metamorphosis to pupae, and their eventual emergence as adults were made in comparison with larvae fed on a mixture of 100μM selenate and 100μM arsenate and larvae fed with a control diet without selenate and arsenate. Insect rearing and details of materials and methods were similar to the arsenic study in chapter 4.

6.2.2 Determination of Total Selenium in Insects using Atomic Absorption Spectroscopy

Surviving 5^{th} instar larvae, pupae and adults, ten from each stage, were sampled for total selenium analysis. Selenium analysis was done using 5 larvae, 5 pupae and 3 adults as described in Chapter 2. Larvae from the combined treatment did not survive for further examination.

6.2.3 X-ray Absorption Spectroscopy

Selenate treated insects were taken for XAS studies as follows: larva (ten 5^{th} instar larvae, 2 larvae/replicate); pupa (ten pupae, 2 pupae/replicate); and adult (five males and five females of newly emerged moths, 1 moth/replicate). Larval exuvia and frass were also collected for analyses.

X-ray absorption spectroscopy was carried out at the Stanford Synchrotron Radiation Laboratory (SSRL) on beamline 9-3 as described in Chapter 2. Spectra of

dilute aqueous solutions of standard selenium species were also collected, as previously described (Chapter 2). The fractional contribution of a model spectrum to the sum is equal to the fraction of total selenium present in that chemical form in the sample.

6.2.4 Micro X-ray Fluorescence Imaging and X-ray Absorption Spectroscopy Imaging

Micro X-ray fluorescence imaging (μ-XRF) and chemically specific imaging were conducted on selenate (100 μM) fed 3rd instar bertha armyworm larva using the methods previously described in Chapter 2. The distributions of other metals such as copper, iron, manganese and zinc were also monitored simultaneously with those of selenium, to observe possible co-association of these metals with selenium.

6.3 Results and Discussion

6.3.1 Toxicity of Selenate

Dietary exposure of 100 μM of selenate impaired the larval survival, and larval and pupal development of bertha armyworm compared to the control experiment (Table 6.1). Furthermore, the larvae developing on selenium treated diet were smaller in size and more inactive than the larvae developing on control diet. However, insects did not show any morphological abnormalities to selenate treatment. Interestingly, larvae fed on the same concentration of arsenate showed less toxic effects on survival and development than selenate (Table 6.1, Chapter 5), at the level of 100 μM (except for pupal weight).

Relatively few studies are available on the effect of metals or metalloids on animal growth. The observed increase in the development time of selenate treated larvae would decrease the rate of population increase and expose the remaining larvae to additional mortality factors from rain and other weather conditions, and

predators. Such increase in development time may impair the population fitness of organisms.

6.3.2 Toxicity to Selenium and Arsenate Combined Treatment

None of the larvae treated with the combined treatment of selenate and arsenate survived beyond the 3nd instar stage. In this experiment, arsenic and selenium did not antagonize each other's toxicity in early larval instars, but show additive or even synergistic detrimental effects. Larvae under this treatment showed more signs of toxicity than the larvae in selenium and arsenic separate treatments. Larvae in combined treatments were less mobile, smaller and took longer time to complete a instar stage suggesting a synergistic effect from selenate and arsenate combined treatments.

Table 6.1 Effect of Selenate and Arsenate on Bertha Armyworm

Treatment	Larval Survival (%)	Days to pupation Larvae-Pupae	Pupal Weight (g)	Days to adult emergence (Pupae- Adults)
Selenate	52.0±1.2^{c}	34.0±0.4^{a}	0.35±0.01^{a}	28.4±0.2^{a}
Arsenate	79.0±1.0^{b}	27.4±0.2^{b}	0.35±0.01^{a}	23.4±0.2^{a}
Selenate and Arsenate mixture	NA	NA	NA	NA
Control	92.0 ±1.2^{a}	27.0 ±0.0^{b}	0.35±0.01^{a}	21.2±0.2^{c}

Table shows mean ± standard deviation for n=5 replicates (20 larvae per replicate). Means followed by the same letter (^{a,b}) within a column are not significantly different (Tukey's test, p≤0.05). Insects were treated with 100 µM of selenate, 100 µM of arsenate and 100 µM of selenate and 100 µM of arsenate mixture. NA- larvae did not survive for observations

6.3.3 Determination of Total Selenium using Atomic Absorption Spectroscopy

According to the atomic absorption spectroscopy results, larvae, pupae and adult accumulated total selenium levels of 8.2±1.0, 5.6±0.4 and 3.2±0.7 $\mu g.g^{-1}$(dry weight), respectively. All developmental stages of bertha armyworm showed selenium levels above 3 $\mu g.g^{-1}$ which is the threshold level that exert toxicity in some predators (Chapter 3). Previous studies demonstrated that adults of terrestrial insects can bioaccumulate selenium (6). The ability of the larvae to transfer selenium to pupae and adults provides a source of selenium in the habitats away from the contamination site.

In order to compare the molar amounts of selenium and arsenic accumulated in insects, total selenium and arsenic levels in insects given in Table 6.2 were converted to $nmol.g^{-1}$. Larvae, pupae and adults in 100 μM selenate treatment accumulated 104, 71 and 41 $nmol.g^{-1}$(dry weight) of selenium, respectively. When the level of arsenic in insects given in Table 5.3 were converted to molar amounts, 100 μM arsenate fed larvae accumulated 240, 147, 93 $nmol.g^{-1}$(dry weight) of arsenic in larvae, pupae and adults, respectively. When compare the molar amounts, larvae, pupae and adults accumulate arsenic nearly twice the amount of selenium indicating that these insects can incorporate more arsenic than selenium without showing many ill effects.

6.3.4 Selenium Speciation

Selenium K near-edge spectra of standard selenium compounds and spectra of larvae, pupae and adult stages are given in Figure 3.2 (Chapter 3) and Figure 6.1, respectively. These results show that the chemical form of selenium is changed as it passes from food to larvae, pupae and adults. Selenate in the diet is taken up by the larvae and biotransformed into organic selenium. Quantitative analysis of these spectra (Table 6.2) modeled as selenomethionine (R-Se-R) and diselenides (R-Se-Se-R). During the development from larva, pupa to adult, the fraction of R-Se-Se-R

gradually decreases while R-Se-R increases. Emerging adults predominantly carry R-Se-R.

Previous study with aquatic insects showed a significant fraction of selenite in the larvae (2, Chapter 3). Moreover, pupae of caddisflies (*Rhyacophila* sp.) showed a significant fraction of trimethylselenonium like species, which was absent in bertha armyworm pupae. However, the presence of trimethylselenonium in pupa has been reported from larvae fed selenate-irrigated plants grown in a laboratory study (7). These variations in speciation could be due to the different uptake routes of selenium and/or development stage of the insect. Uptake from alfalfa plants may include organic selenium forms. The presence, absence or any changes of trimethylselenonium like species during pupal development, worth further studying.

Excretion of selenium in exuvia and frass was evident. The spectra of exuvia also fit with R-Se-R, and R-Se-Se-R indicating excretion of organic selenium. Analysis of frass spectra showed that some selenate is concentrated in the fecal pellets (51%). Also, a significant fraction (49%) of selenium in the frass is expelled as R-Se-R. In mammals, selenium is thought to be excreted by incorporation into proteins or by methylation and being removed as methylselenol and trimethylselenonium in urine, while dimethylselenide is released by respiration (8). Previous study suggested that arsenic may bind with chitinous peritrophic matrix of the bertha armyworm larvae and excreted with frass and exuvia. Formation of such complexes with selenate was not indicated in this study. Due to essential nature of selenium, insects may allow absorption of adequate levels of this element. It is possible that the peritrophic matrix in the midgut of insects may not participate binding of selenium and therefore, selenate in frass is excreted unchanged.

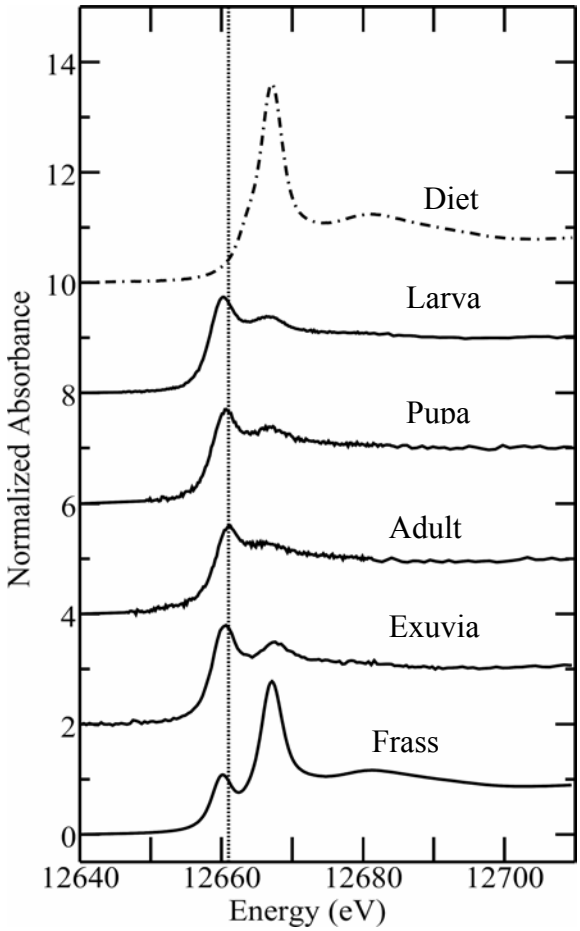

Figure 6.1 Selenium K near-edge spectra of diet, insect samples (larvae, pupae and adults) and excretory matter (exuvia and frass).

Table 6.2 Percent of selenium species in the samples

Sample	Total Selenium Level[A] μg g^{-1} (dry weight)	Percentage of Selenium[B] as:		
		Selenate SeO_4^{2-}	R-Se-R	R-Se-Se-R
Larva	8.2±1.0	0	4(3)	96(2)
Pupa	5.6±0.4	0	34(2)	66(2)
Adult	3.2±0.7	0	93(3)	7(3)
Larval exuvia	0	0	32(2)	69(1)
Frass	0	51(2)	49(3)	0

[A] Total selenium measured using atomic absorption spectroscopy (mean ± standard deviation, n=2).
[B] Percentage composition of selenium chemical species, derived from the contributions of standard spectra to the fit of Se K near-edge X-ray absorption spectra of insect samples. Selenate, selenite, selenides, diselenides, trimethylselenonium and elemental selenium are represented by SeO_4^{2-}, SeO_3^{2-}, R-Se-R, R-Se-Se-R, R_3Se^+ and Se^0, respectively. Figures in parenthesis show three times the estimated standard deviation of each parameter in the fit, derived from the covariance matrix.

6.3.5 Localization of Selenium Species and Other Elements using X-ray Absorption Spectroscopy Imaging

Maps of selenium and other elements were taken on third instar bertha armyworm larvae. Energies used for selenium imaging were corresponding to the X-ray absorption of standard solutions of selenate at 12667.3 (chemical form in the diet) and organic selenium in the larvae at 12661.0 eV (edge energy for selenomethionine standard). Figure 6.2 shows images of bertha armyworm larva at 20 μm resolution to give an overview of the entire organism. Blue in all three

figures indicate the X-ray transmittance, which gives an indication of the amount of material in the projection. Fig. 6.2a shows the localization of organic selenium, which is the predominant form of selenium in the larvae and selenate, which is the chemical form in the diet. Distribution of organic selenium differs from the distribution of organic arsenic species observed in Chapter 5. Organic selenium was detectable in the midgut area of the larvae with distinctly high localization towards the posterior region of the midgut (Figure 6.2 a). In addition, organic selenium distributed in other parts of the body such as periphery of the head and legs and posterior part of the body suggesting this form is broadly incorporated into the tissues.

In contrast to arsenate, which was diffusely distributed throughout the larval body in Figure 5 d,h, selenate appeared to be associated with the a fecal pellet at the end of the digestive tract (Figure 6.2 a). Traces of organic selenium and other elements such as copper, iron, zinc and manganese are also present in the fecal pellet. Copper (Figure 6.2 b), iron, zinc (Figure 6.2 c) and manganese localization within the larva closely follow the distribution of these elements in midgut, mandibles and pigment areas in arsenate treated larva. The copper area outside the alimentary canal on the mid ventral side of the larval body in arsenate treated larva was more pronounced in Figure 6.2 b. Organic selenium is also localized in this area (Figure 6.2 a). These images suggest that larvae fed with 100 μM of selenate do not retain selenium as selenate in the body. Observations on the presence of selenate in frass in the image agree with the bulk spectra obtained from frass which indicates 51% of the selenium as selenate in frass (Table 6.2).

Previous studies indicate that concentration of trace elements in the insect midgut varies with the insect taxa (genera) and the element under consideration (9). In some genera of Chironomidae, cadmium was found largely on the anterior region of the midgut, but in some genera this element was mainly confined to the

posterior region (10, 11). The reason for the differences in distribution pattern is not known.

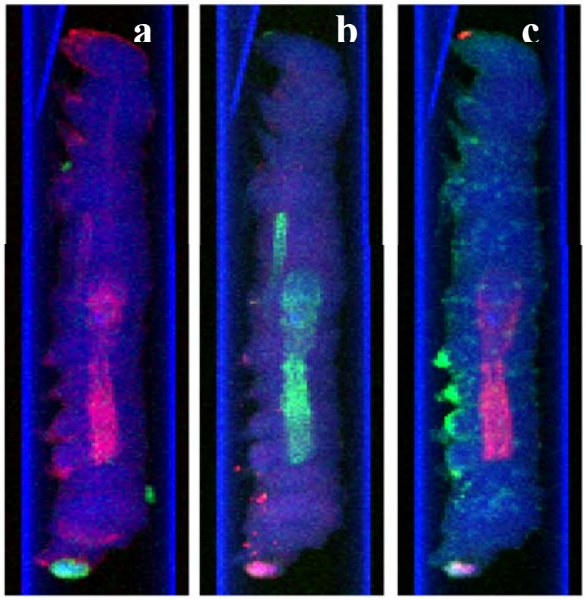

Fig. 6.2 X-ray fluorescence imaging of metals in selenate fed bertha armyworm larvae. a) organic selenium (red) and selenate (green), b) iron (red) and copper (green), c) Zinc (red) and manganese (green) a-c) transmittance is shown in blue. Orientations are lateral, head up and mouth facing left. Intensity scales are arbitrary

6.4 Conclusions

The present study shows that selenium in the diet directly affects survival of bertha armyworm larvae, and development of larvae and pupae. Selenium body burdens in larvae, pupae and adults gradually decreased during metamorphosis, indicating that regulation of selenium accumulation varies with the development stage. However, all stages of the insects carry selenium levels potentially harmful to some predators.

Bertha armyworm larvae are able to biotransform inorganic selenium in the diet into organic forms. The chemical form of selenium responsible for toxicity observed in this study is mainly represented by selenides and diselenides. Elimination of selenium in the form of selenate and organic selenium in frass and organic selenium in exuvia may help to alleviate selenium bodyburdens in successive developmental stages.

Localization of selenium within the larva was different from localization of arsenic observed in Chapter 5. Organic selenium was higher in posterior part of the midgut than in other regions. Selenate taken from the diet was found at the end of the digestive tract most probably associated with a fecal pellet indicating a possible excretion route.

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7. EFFECTS OF SELENIUM AND ARSENIC ALONE AND IN COMBINATION ON WASP PARASITOIDS OF BERTHA ARMYWORM

7.1 Introduction

Parasitism and parasitoidism are important factors in regulating insect populations. Parasitoidism is unique to insects and combines attributes of both predation and parasitism (1). Parasitoidism is found in many insect orders including Hymenoptera, Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera and Strepsiptera (1). However, 80% of the known parasitoid species belong to the order Hymenoptera. Generally, larva of the parasitoid feeds and develops inside a living host that it eventually kills (1). In contrast, parasites are adapted to utilize the host for long periods without killing it. In both situations, parasite and parasitoid associations are coevolved to utilize certain host-parasite or host-parasitoid associations. Because of this host specificity, parasites and parasitoids are considered as important regulators of their hosts and have been primary agents in biological control programs (3**).**

Metals or metalloids may change host-parasitic relationships by affecting either the host or the parasitoid, or both. There are fundamental differences in metal or metalloid uptake by hosts and parasitoids. Until recently, only a few studies have dealt with the influence of metals or metalloids on hymenopteran parasitoids and host-parasitoid relationships (4-6). It is suggested that some herbivorous insects living in polluted areas may benefit from the reduction of parasitoid populations (7). However, during investigations of a host-parasitoid relationship, cadmium and lead of the host *Ceratitis capitata* (Diptera: Tephritidae) did not alter the development of the parasitoid *Coptera occidentalis* (Hymenoptera: Diapriidae) (8). Only one study has examined the effect of selenium on the host parasitoid relationship (4). Under laboratory conditions, selenium rich alfalfa fed beet

armyworm was parasitized by a larval parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). In this study, selenium delayed the parasitoid's development and lowered the pupal weight compared to wasps emerging from hosts on control plants (4). The chemical form of selenium in hosts and parasitoids was also studied. The effects of arsenic on parasitoids are not known.

In order to understand the effects of metals or metalloids, it is necessary to study changes occurring in other trophic levels such as parasitoids as well because the ecosystem structure and function depend on interactions among all trophic levels. Very little progress has been made to evaluate the impacts of metals or metalloids when they occur in the environments in mixtures. Although antagonistic effects of certain chemical forms of selenium and arsenic are known in mammals (9), the effects of combined action of selenium and arsenic on other animals are not known. The aim of this study is to assess the possible effects of arsenate, selenate and a combination of arsenate and selenate on insect host-parasitoid interactions. X-ray Absorption Spectroscopy was used to identify the speciation of selenium and arsenic in larvae, pupae and adults stages of the parasitoids. Possible excretion of these elements through pupal cocoons of the parasitoids was investigated**.**

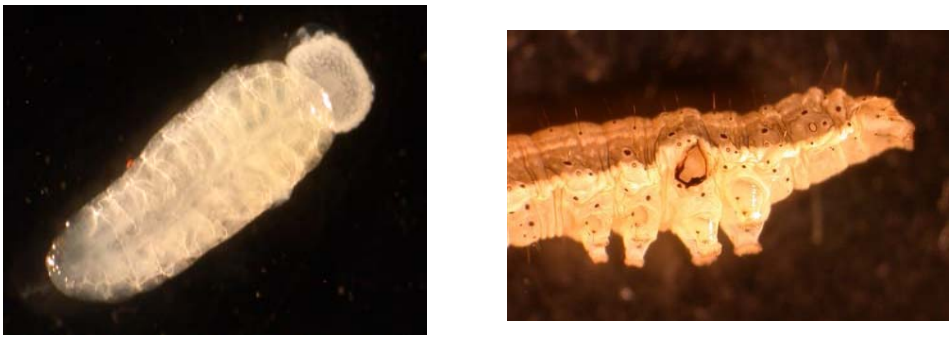
7.2 Material and Methods

7.2.1 Parasitoid *Microplitis mediator* (Haliday): Biology

Microplitis mediator (Haliday) [Hymenoptera: Braconidae], is a larval parasitoid of bertha armyworm. This parasitoid was introduced from Europe to control bertha armyworm in Canada.

Arthur and Mason (10) described the biology and behavior of *M. mediator* attacking bertha armyworm larvae. Females of *M. mediator* attack the first three larval instars of the bertha armyworm. The 4^{th} and 5^{th} instars are able to repel the attack. A single egg is deposited by the parasitoid into the host's hemocoel (blood)

by piercing through the cuticle with the ovipositor. Eggs hatch within 18-56 h after oviposition and develop inside the body of the host. All larval instars of the parasitoid feed on the host's abdominal tissues. As the first instar ages, a bubble-like anal vesicle develops and the larval body enlarges greatly (Figure 7.1a). The third instar parasitoid larva leaves the host through a hole cut in the side of the fifth or sixth abdominal segment of the bertha armyworm larva (Figure 7.1b). Immediately after exiting from the host, parasitoid larvae pupate in a light brown silken cocoon. The adults emerge by cutting off one side of the cocoon. Larvae take about 2.5 weeks to develop to maturity and emerge from the host. Typically, host larvae molt twice and die at the 4^{th} instar stage after the emergence of the parasitoids.



a **b**

Figure 7.1 a) Late stage first instar larvae of *M. mediator* b) Bertha armyworm larvae showing the exit hole of the parasitoid larvae

7.2.2 Rearing of Parasitoids

Adults *M. mediator* were confined in wooden cages with two openings that were covered with nets. They were provided with 10% sugar and honey solution and maintained at 21 ± 1 ºC. Initially, 400 bertha armyworm larvae were reared to the 2nd instar on a modified Bucher Bracken diet prepared in absence of added selenate or arsenate (Chapter 5). Each 2nd instar larva was taken from a paintbrush and offered to adult female wasps of *M. mediator*. Parasitized host larvae were then transferred to diet cups containing the treatments: 1) 100 μM arsenate; 2) 100 μM selenate; 3) a mixture of 100 μM arsenate and 100 μM selenate; 4) arsenate- and selenate-free control. Each treatment consisted of 100 larvae; four larvae were placed in one cup, and were replicated five times with five cups per replicate. The number of parasitoid pupae emerged from the larvae, the number of parasitoid adults emerged from pupae and the time (days) to pupal and adult emergence were recorded.

Nine days after parasitization, bertha armyworm larvae were dissected to obtain parasitoid larvae. At this stage, parasitoid larvae have attained their maximum size, which is shown in Figure 3. Ten such larvae were collected, mounted in 2 mm sample cuvettes and frozen in liquid nitrogen for XAS analysis as described in Chapter 2. Ten parasitoid pupae enclosed in their cocoons, thirty empty cocoons, and 20 adult parasitoids were also prepared for XAS. Additionally, five unparasitized 5th instar bertha armyworm larvae feeding on selenium and arsenic combined treatments from 2nd instar stage were prepared for XAS analysis. Host larvae that are not parasitized, but fed on selenium and arsenic separate treatments from neonate (1st instar) stage were examined in Chapter 5 and 6. These results are included here as well.

7.3 Results and Discussion

7.3.1 Toxicity to Parasitoids

Effect on parasitoid larval development, pupal development and adult eclosion was examined. Days to pupation (time taken by 50% of the larvae to reach pupation), larval survival (% number of larvae pupated), pupal weight, days to adult emergence (time taken by 50% of the pupae to reach adult stage) and pupal survival (% number of adults emerged), were analyzed by ANOVA and the results are given in Table 7.1.

Table 7.1 Effect of selenate and arsenate alone and in combination on the Parasitoids of bertha armyworm

Treatment	Larval Survival (%)	Days to Pupation	Pupal Survival (%)	Pupal Weight (mg)	Days to adult emergence
Selenate	86±1[b]	16±0[a]	97±2[a]	3.06±0.04[ab]	5±0[a]
Arsenate	94±1[a]	16±0[a]	100±0[a]	2.32±0.04[b]	5±0[a]
Selenate and Arsenate	72±2[c]	16±0[a]	97±2[a]	2.28±0.02[b]	5±0[a]
Control	97±2[a]	16±0[a]	99±1[a]	4.32±0.06[a]	5±0[a]

Larval or pupal survival (%), pupal weight (mg) Means followed by the same letter are not significantly different (Tukey's test, p≤0.05)

In this experiment, dietary treatments of selenate and arsenate, alone and in combination, had no significant effect on days to pupation, pupal survival and days to pupal emergence of parasitoids. Larval survival was significantly lower in selenate treatment and in selenate and arsenate co-treatment than in arsenate

treatment. Larvae developing in control had 97% survival. In parasitoids, exposure to arsenate, either alone or in combination with selenate, resulted in significant pupal weight reduction compared to the other treatments, with the combined treatment and arsenate treatments showing the largest effect. Effect on selenium treated hosts was intermediate. In a previous study, selenium in the diet of beet armyworm (*Spodoptera exigua*) reduced the pupal weight and slowed the development, but did not influence the survival of the larval parasitoid *Cotesia marginiventris* (4). However, host larvae showed no change in pupal weight, whereas the wasp shows the largest effects in this category.

Arsenic treated parasitoids were the least affected in this study. This observation supports earlier findings, which indicate resistance of parasitoids to host metals (8). The fact that selenium is somewhat toxic to the hosts, but not to the parasitoids could be due to the lower level of selenium in parasitoid tissues. However, due to small sample size, no data were taken on the level of selenium and arsenic in parasitoids.

7.3.2 Survival of the 2nd Instar Bertha armyworm Larvae under Selenate and Arsenate Combined Treatment

In this experiment, host larvae were introduced to the selenate and/or arsenate treatments at the 2nd instar stage. Despite the fact that larvae were additionally stressed by parasitization, bertha armyworm larvae in this experiment were less affected by the treatment mixture than the neonate larvae in the previous experiment, even though the diet was the same concentration. Previous findings in Chapter 5 revealed that neonate larvae were not able to complete the development beyond the third instar stage under the combined selenate and arsenate treatment. In general, neonates have to face various obstacles to establish themselves in a new life and as a result, the requirements of later instars may be different from the neonates (11). The reason behind the observations on susceptibility of the neonate

larvae and tolerance of the second instar larvae to the selenium and arsenic combined treatment remains to be understood. Detailed observations on the animal and chemical speciation of selenium and arsenic may explain this.

7.3.3 Speciation of Selenium in Hosts and Parasitoids

The determination of selenium and arsenic speciation in *M. mediator* revealed trophic transfer of these elements from bertha armyworm larvae to the parasitoids. Selenium K near-edge spectra of parasitoids developing in selenate treated hosts and selenate and arsenate co-treated hosts are given in Figure 7.2. Edge fitting results of these spectra are given in Table 7.2.

Host larvae treated with selenium were found to contain organic selenium (Chapter 6, Table 7.2). However, host larvae receiving selenium and arsenic combined treatments retained some selenate (26%). Under both treatments, host larvae were able to excrete selenate in frass. Higher fraction of selenate was detected from frass collected from larvae in combined treatments (Chapter 6, Table 7.3). Selenate near edge spectra from frass did not show any modifications to the chemical form during excretion. Due to the essential nature of selenium, it is possible that insects are adapted to absorb and utilize certain amount of selenium and excess is excreted unchanged via frass. Properties of peritrophic membrane of larva may be examined in this regard to see any differences in binding capacities of these two metalloids alone and in combination.

Selenium in parasitoids developing in selenate-treated hosts and selenate and arsenate treated hosts was mainly in organic forms with more than 60 % of selenium in the form of R-Se-Se-R. Parasitoid larvae, which received the selenate only treatment, contained a small fraction of selenate (18%). Only a trace (2%) of selenate was detected in parasitoid pupa. In an earlier study, Vickerman et al. reported that parasitoid pupa developed from selenate treated hosts contain organic selenium (4). Parasitoids from the selenate and arsenate combined treatment had

more than 26% of selenium as selenate. Emerging parasitoid adults from selenium treatment carry 100% of selenium as R-Se-R. Parasitoid adults from a previous study (4) had 75% of selenium as R-Se-R, and 25% of trimethylselenonium like species. However, significant fraction of trimethylselenonium like species was not detected from adults obtained in this study. Spectra taken from the mixture fed parasitoid pupae were noisy probably because of the low concentration of elements and therefore, reasonable analysis could not be obtained. In mammals, a specific detoxification molecule [$(GS)_2AsSe^-$] is formed when selenite and arsenite are given together. However in this study, $(GS)_2AsSe^-$ was not detected from host insects or from their parasitoids when treated with selenate and arsenate.

Selenate in the parasitoid larvae may come from larval excretory matter retained within the larval body. A key feature of the development of parasitic wasp larvae is the separation of the midgut and hindgut until just prior to pupation (1). This way, parasitoid larva is unable to void waste products and indigestible food into the host body (1) and deteriorate the host. Storage areas within the parasitoid larvae are not known.

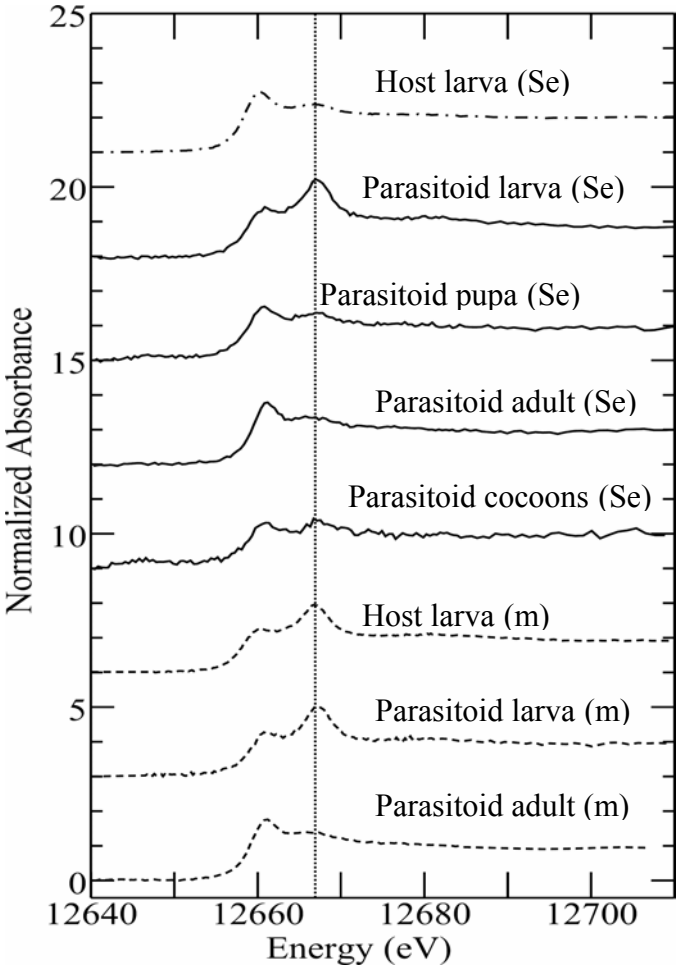


Figure 7.2 Se K near edge spectra of parasitoids reared on selenate (Se) and selenate and arsenate mixture (m) treatments in comparison with the spectra from the host larvae. Spectra taken from the mixture fed parasitoid pupae were noisy and they were not used in the analyses.

Table 7.2 Percent selenium species in insects received selenium (Se) and a mixture of selenium and arsenic (m)

Sample	Selenate		R-Se-R		R-Se-Se-R	
	Se treated	**m treated**	**Se treated**	**m treated**	**Se treated**	**m treated**
Host larva	**0**	26(1)	**4(3)**	0	**96(2)**	74(2)
Parasitoid larva	**18(2)**	26(6)	**22(2)**	51(1)	**60(2)**	23(1)
Parasitoid pupa	**2(1)**	NA	**38(2)**	NA	**60(1)**	NA
Parasitoid cocoons	**0**	NA	**38(2)**	NA	**62(2)**	NA
Parasitoid adult	**0**	0	**100(6)**	91(1)	**0**	9(1)

Edge fitting results of insect samples. Values derived from percentage contributions of spectra. Figures in parenthesis show three times SD. Spectra from combined treatment received parasitoid pupa and parasitoid cocoons were too noisy therefore the results are not available (NA). Figures in bold indicate the results of selenate only treatment.

7.3.4 Excretion of Selenium by Hosts and Parasitoids

The empty cocoons left by parasitoids when they emerge into adults were examined for selenium. They were compared with excretory matter (exuvia and frass) of bertha armyworm larvae reported in Chapter 6 and exuvia and frass of hosts receiving the arsenate and selenate combined treatments. X-ray Absorption near edge spectra of exuvia and frass of bertha armyworm and empty parasitoid pupal cocoons are shown in Figure 7.2. Results of the edge fitting analysis are given in table 7.3.

Although selenate was not detected from selenium fed host exuvia, selenium and arsenic hosts receiving the combined treatment receiving hosts were

able to excrete some selenate in exuvia. Exuvia from both treatments contained organic selenium in the form of R-Se-R and R-Se-Se-R.

Table 7.3 Percent selenium species in host excretory matter and parasitoid cocoons

Sample	Selenate		R-Se-R		R-Se-Se-R	
	Se treated	**m treated**	**Se treated**	**m treated**	**Se treated**	**m treated**
Host exuvia (Chapter 6)	**0**	20(2)	**31(2)**	29(3)	**69(1)**	51(2)
Host frass (Chapter 6)	**51(2)**	63(2)	**0**	20(3)	**49(3)**	17(1)
Parasitoid cocoons	**0**	NA	**61(3)**	NA	**39(1)**	NA

Edge fitting results of insect samples. Values derived from percentage contributions of spectra. Figures in parenthesis show three times SD. Se- selenium only treatment, m- selenate and arsenate mixture treatment

Selenium was detected from braconid parasitoid (Hymenoptera: Braconidae) cocoons collected from the field (Chapter 4) as well as parasitoids reared under laboratory conditions (Table 7.3). Many parasitic wasps construct silken cocoons to pupate. The cocoon is generally considered to be protective and prevent the pupa from desiccation, predation and harmful substances. A possible excretory function of the cocoon is suggested but not fully understood (1). However, detection of selenium in the cocoons indicates that larvae are able to excrete selenium through pupal cocoons.

7.3.5 Speciation of Arsenic in Insects and Excretory Matter when Treated with Arsenate Alone and in Combination with Selenate and Arsenate

Arsenic K near-edge spectra of host larvae treated with arsenate and their parasitoids are given in Figure 7.3. Edge fitting results are given in Table 7.4. Initially, host larvae treated with arsenate treatment and selenate and arsenate combined treatment had more than 75% of arsenic as As^{III}-*tris*-glutathione. Parasitoids carry the majority of arsenic as As^{III}-*tris*-glutathione. In pupae, 100% of arsenic was detected as As^{III}-*tris*-glutathione. Adults of this treatment had 81% As^{III}-*tris*-glutathione, and 19% arsenite.

In addition to 62% of arsenic as As^{III}-*tris*-glutathione, parasitoid larvae contain a form of arsenic bound to two sulfur atoms modeled as As^{III}-*di*-glutathione. A small fraction of arsenic as arsenate was detected in parasitoids treated with arsenate (9%) and selenium and arsenate treatments (5%). More than 80% of arsenic in the adult moths can be modeled as As^{III}-*tris*-glutathione. As indicated in the chapter 5, As^{III}-*tris*-glutathione may be harmless to the host as well as parasitoids studied here.

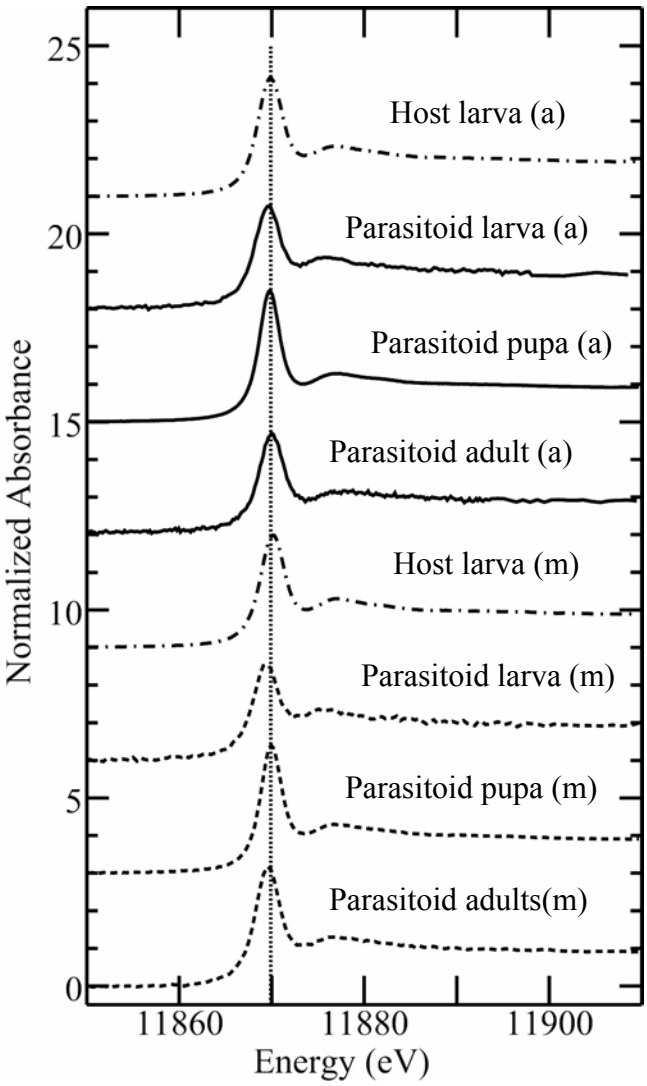


Figure 7.3 As K near edge spectra of larvae reared on arsenate (a) and arsenate and selenate combined treatments (m) and their parasitoids.

Table 7.4 Percent selenium species in insects received arsenate (a) and arsenate and selenate mixture (m)

Sample	Arsenate PH-9		Arsenite		As^{III}-*tris*-glutathione	
	a	**m**	**a**	**m**	**a**	**m**
Host larva	0	0	**11(2)**	24(3)	**89(2)**	76(3)
Parasitoid larva	**9(1)**	5(3)	**0**	0	**91(1)**	62(1)+33(1) [b]
Parasitoid pupa	0	0	**0**	14(3)	**100(1)**	86(3)
Parasitoid adult	0	0	**19(1)**	0	**81(3)**	100(1)

Edge fitting results of insect samples. Values derived from percentage contributions of spectra. Figures in parenthesis show three times SD. 33(1)[b] indicates 33% of another component modeled as As^{III}-*di*-glutathione. Figures in bold indicate results of arsenate only treatment (a). Results of the arsenic and selenium combine treatment are given under m.

7.3.6 Excretion of Arsenic by Hosts and Parasitoids

Excretion of arsenic in arsenate treated bertha armyworm is described in chapter 4. Spectra are shown in again in Figure 7.4 along with the spectra of arsenic and selenium combined treatments. It is important to note that major fraction (more than 60%) of excretory products from both treatments is in the form of arsenate at pH 0 with arsenic coordinated with six oxygen atoms. Detailed description of 6 coordinated arsenic species is described in Chapter 5. Exuvia of host larvae received arsenate and selenate combined treatments differ slightly from exuvia of arsenate treated larvae in that As^{III}-*tris*-glutathione was not detected from combined

treatments. Bertha armyworm larvae from arsenate treated experiment excreted frass containing arsenate species at pH 9 (20%) and pH 0 (60%). Frass from combined treatments had 100% of arsenic as pH 0 species (Figure Table 7.5). It is interesting to note that empty cocoons of the hosts treated with arsenate and empty cocoons of the parasitoids in both treatments had 100% of arsenic as As^{III}-*tris*-glutathione.

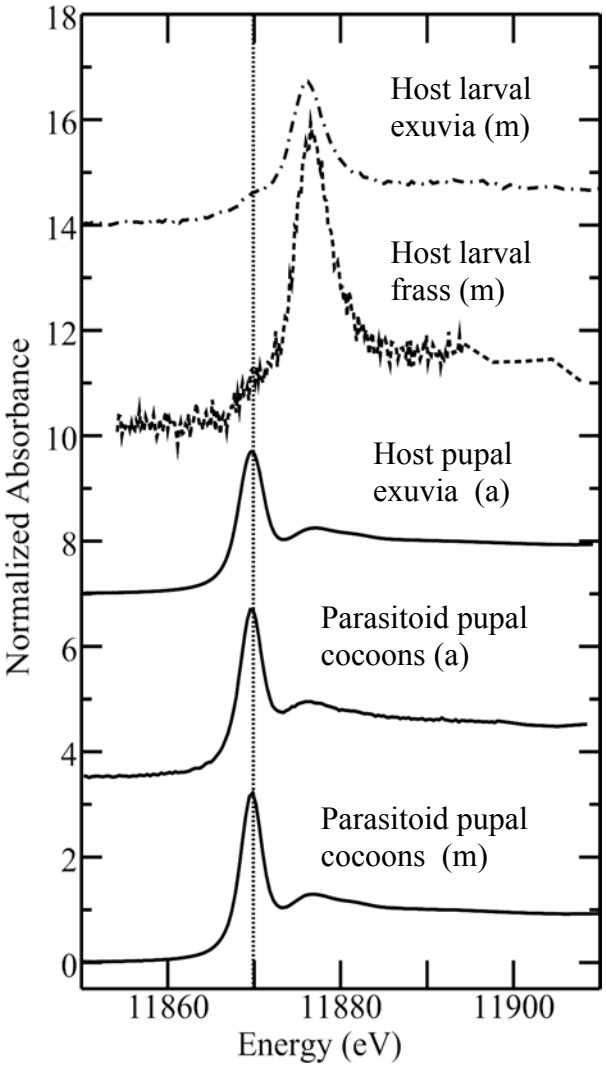


Figure 7.4 As K near-edge spectra of parasitoid pupal cocoons in comparison with the excretory products of the hosts when treated with arsenate alone (a) and with selenate and arsenate mixure (m)

Table 7.5 Percent arsenic species in exuvia and frass of hosts treated with arsenate and arsenate and selenate combined treatments and parasitoid cocoons

Sample	Arsenate (pH 9)		Arsenate (pH0)		As^{III}-*tris*-glutathione	
	a	**m**	**a**	**m**	**a**	**m**
Host exuvia	0	25(3)	**66(1)**	65(1)	**34(1)**	0
Host frass	**20(3)**	0	**60(3)**	100(1)	**20(1)**	0
Host cocoons	0	NA	**0**	NA	**100(1)**	NA
Parasitoid cocoons	0	0	**0**	0	**100(1)**	100(1)

Edge fitting results of insect samples. Values derived from percentage contributions of spectra. Figures in parenthesis show three times SD. Results in bold figures indicate arsenate only treatment. NA- not available

7. 4 Conclusions

Results from the present study broaden the knowledge on transfer of trace metals such as selenium and arsenic through host-parasitic interactions. Accumulation of selenium in bertha armyworm larvae reduced the survival of parasitoid larvae under selenium and selenium and arsenic combined treatments. Arsenate alone treatment was relatively less toxic to the parasitoids. Although selenate was not detected in the selenium treated hosts, presence of selenate in selenium and arsenic treated hosts and parasitoids is interesting. These observations and warrant further attention.

As^{III}-*tris*-glutathione appears to be the main arsenic species in the host as well as in the parasitoids. Frass and exuvia of hosts treated with arsenate and

selenate and arsenate combined treatment employ 6 coordinated arsenic species during arsenic excretion. The novel six coordinated arsenic species found in arsenate treated larval excretory matter is found in exuvia and frass collected from selenate and arsenate combined treatment fed larvae as well. Empty cocoons left by parasitoid pupae after adult emergence entirely contain As^{III}-*tris*-glutathione.

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7.5 References

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8. CONCLUSIONS AND FUTURE DIRECTIONS

8.1 Conclusions

Insects constitute a substantial portion of the aquatic and terrestrial food webs. Studies presented here illustrated the ability of insects to accumulate selenium and arsenic, and biotransform into different chemical forms.

Elevated levels of selenium in stream environments near Hinton, Alberta, aid in bioaccumulation of selenium in insects and biofilm. Although some studies suggest that organisms living in lotic (fast flowing) aquatic environments are poor accumulators of selenium, results on stream insects and biofilm examined in this study agree with other studies, which showed high bioaccumulation of selenium in lotic habitats.

The high sensitivity of X-ray absorption near-edge spectra has been shown and used for analyses of field-collected insects and biofilm samples from two selenium-contaminated streams. Organic forms accounted for more than 85% of the selenium in all nymphal, larval and pupal stages of the insects examined. These insects mainly contain selenium in the form of selenides (R-Se-R). Less than 15% of the selenium was detected as selenite with higher percentage in herbivorous insects and detritivorous insects (9-15%) than in predatory insects (2-7%), indicating a slight magnification of organic selenium in predators and detritivores. These insects are food for many insectivorous predators such as fish and birds and total selenium levels in insects (more than 4 $\mu g\ g^{-1}$ dry weight) exceeded the lower toxicity threshold level reported for fish and birds (3 $\mu g\ g^{-1}$ dry weight). Since the common R-Se-R (selenomethionine) and selenite can exert high toxicity levels in predators, speciation results indicate a potential risk to sensitive fish and birds living in this stream environment. Trimethylselenonium-like species observed in

caddisfly pupa can act as precursor to the synthesis of volatile forms of selenium indicating a pathway of releasing selenium from the water.

Selenium in some environments has led to the evolution of insects that are adapted to feed on selenium hyperaccumulating plants. Leaf folding larvae (Diptera) were adapted on immature leaves and seed beetles (Coleoptera) utilized seeds of *A. bisulcatus* for shelter and food. Lycaenid caterpillar (Lepidoptera) found on leaves did not show any preference for a plant part. All insects examined carry selenium mainly in the form of organic selenides (R-Se-R).

Laboratory studies conducted here evaluated the toxicity and biotransformation under controlled conditions. This way, confounding effects of other environmental factors were avoided by isolating the treatments. The effects of larval uptake of selenate and arsenate alone and in combination on the development of the insect and insect parasitoids were studied. The role of various excretory pathways was closely examined.

Under laboratory conditions, selenate is more toxic to insects than arsenate at the same dietary concentration. In arsenate treated insects, arsenic accumulated within the body as conjugates with GSH or other thiols. Methylated arsenic forms commonly seen in mammals were not observed in this study. The observations on the novel six coordinated arsenic species excreted in exuvia and frass of insects improve our understanding on behavior of arsenic within organisms and ability of insects to tolerate arsenate toxicity. The chemistry of arsenate when mixed with a di-hydroxyl such as catechol or glycerol under very low pH is shown. Since exuvia shed by insects may become food for the insects as well as detritivores in the environment, it is possible that six coordinate arsenic species may contribute to the food chain as well. Further studies on the occurrence of six coordinate species in insects under field situations and its potential toxicity to detritivores are important in understanding its effects on the environment. The possibility of binding arsenic to the chitinous exoskeleton of insects worth exploring. Since polysaccharides such

as chitin and chitosan are part of the exoskeleton of many arthropods including crustaceans and insects, storage and excretion of elements in chitin and chitosan would be a significant advantage for these organisms living in contaminated environments.

Selenate in the diet was found to prolong larval development, reduce pupal weight and increase the time to adult emergence. It is possible that for insects, threshold values for toxic reactions from organic selenium is much lower than the values for organic arsenic observed in this study, resulting in higher toxicity in selenate treated larvae than in arsenate treated larvae. Similar to the field study, organic selenium constitutes a major fraction of selenium in insects. These organic selenium forms found in insects may exert higher toxic reactions than organic arsenic species. With selenate treatment, there was no evidence for analogous six-coordinate species in frass or exuvia.

Observations on selenium and arsenic combined treatment revealed important information that could improve our understanding on interactions of these elements in insects. Combined treatment was highly toxic to neonate bertha armyworm larvae. However, when given at 2nd instar stage, larvae showed lower toxicity. The involvement of a selenium and arsenic detoxification molecule seen in mammals was not evident in these insects.

No negative impacts of host metal stress on parasitoid development and survival was observed except for pupal weight and larval survival. More than 70% of the parasitoid larval survival was achieved. Parasitized bertha armyworm larvae feeding on artificial diet were exposed to toxic inorganic forms of selenate and arsenate, whereas the parasitoids were exposed mainly to organoselenium and organoarsenic species. Adult hosts and parasitoids contain organic selenium and arsenic, which may be transported to different geographic areas by flying adults. Toxicity to insectivorous predators living away from the point sources may depend on their ability to tolerate these organic forms carried by adult insects.

It appears that selenium and arsenic are eliminated in the larval exuvia and fecal pellets, which may represent important routes by which larvae of Lepidoptera eliminate non-essential and essential elements. Selenium was excreted in exuvia of field collected stonefly (Plecoptera) nymphs as well. Pupal exuvia of Lepidoptera and pupal cocoons left by parasitic hymenopteran pupae after adult emergence carry selenium and arsenic. These enable insects to reduce the body burden during metamorphosis. However, selenium and arsenic forms in exuviae may enter into the food chain through detritivores.

X-ray Absorption Spectroscopy imaging and chemically specific imaging have revealed the region of the midgut where arsenate biotransformation occurs into organic forms. Distribution of arsenic as sulfur coordinated forms was confined to the middle region of the midgut. However, images taken from selenate fed larvae showed more organic selenium in the posterior regions of the midgut than in other places. The distribution of zinc, manganese, copper and iron in selenate fed larvae closely followed the distribution of these elements in arsenate fed larva.

It is evident that insects have been widely studied in relation to metals and metalloid bioaccumulation. However, very few studies used synchrotron techniques to look at metals and metalloids in insects. Studies documented here provide important and unique information, which will be useful in understanding the effects of selenium and arsenic in the environment.

8.2 Recommendations for Future Work

1. The field study near Hinton looked at immature stages of insects that are consumed by fish and birds such as american dipper and harlequin ducks. It is important to study the selenium levels and speciation of selenium in field collected adult insects that spend a terrestrial life, as these flying adults can carry selenium

far away from the point sources. This is especially important, as Hinton is adjacent to the Banff National Park, which is home to more than 200 species of birds.

2. Further research on the ability of the insects to successfully utilize hyperaccumulating plants will improve our understanding on selenium adaptability in organisms. Although due to the small sample size, a total selenium analysis could not be made in this study, it is important to sample more insects to examine the total amount of selenium that these insects can accumulate and carry to other trophic levels. Insects living in nonaccumulator vetches can be tested on accumulator plants for tolerance. Similarly, insects living on accumulator plants can be tested on non-accumulator vetches containing lower selenium. Toxicity or tolerance shown by these insects can be examined with respect to the speciation data. XAS imaging will examine the selenium localization in insects feeding on *Astragalus bisulcatus*. These results can be compared with localization results obtained in non adapted insects to see changes in target organs etc.

3. Present knowledge on the survival and developmental consequences and elevated body burdens of selenium and arsenic will not reflect adult reproductive performance under elevated levels of these elements. Investigations of next generation life parameters such as number of eggs laid, number of larvae survived might elucidate the complete effects of selenium and arsenic. Possibility of transferring selenium and arsenic to insect eggs and to the next generation warrants further studies. X-ray absorption spectroscopy can be used to visualize possible tight association of metals and metalloids with nuclei or other cellular components. Detailed studies on the presence of selenium and arsenic in reproductive organs of adult insects and developing insect eggs would be invaluable in finding answers to the impact of metals and metalloids on the next generation.

4. Neonate mortality on selenate and arsenate combined treatments needs further understanding by comparing the effects with lower concentration of arsenate and selenate in the diet. Detailed observations on the behavior of the animal such as movements, food uptake are essential for understanding the impact. It is evident that under selenium and arsenic combined treatments, larvae retain some selenium as selenate. Ability of neonate to biotransform selenate to organic forms could be further investigated.

5. Accumulation of metals and metalloids in organisms may be used in biomonitoring programs to directly evaluate the impact of elements on the environment and success of remediation strategies. Some insects and mites are able to tolerate metal (Zn and Cu) levels over a wide range of environmental concentrations and become abundant under high metal stress while some sensitive species become locally extinct or become low in population numbers. The abundance of insect and mite taxa are successfully used in predicting sustainability of agricultural systems under heavy fertilizer and other agrochemical input levels. The possibility of using mites and insects as biomonitoring agents in metals and metalloid contaminated sites can be investigated by comparing their uptake, biotransformation and excretion pathways using XAS.

6. The mechanisms of heavy metal biotransformation, especially its transport across the gut membrane and storage in specific cells and cell organelles can be further studied with higher beam resolutions. The specificity of chitin and chitosan to bind arsenic and selenium can be examined and compared. With XAS imaging techniques, it is possible to gain insights into the permeability of insect gut lining. The peritrophic matrix (PM) is a chitin and glycoprotein layer that lines the invertebrate midgut. Although structurally different, it is functionally similar to the mucous secretions of vertebrate digestive tract. PM acts as a physical and

biochemical barrier, protecting the midgut from harmful substances. It is an important target site for insecticide action. The permeability of PM to different metals and metalloids in different insects may be examined with XAS to gain more information on the function of PM which may lead to environmentally friendly insect pest control strategies. Newly developing beamlines, customized for biological research, and which are capable of achieving higher beam resolutions, can lead to major advances in this field.